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# The action of trophic hormones and other substances on the autotransplanted ovary of the ewe

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DECLARATION

This thesis has been composed by myself. The work described herein formed part of a collaborative project to which I made a substantial contribution.

I was solely responsible for the development and application of a competitive protein binding assay for progesterone. Dr. D.T. Baird was responsible for the surgery involved in the preparation of the ewes with transplanted ovaries. I helped in the design and execution of experiments reported in chapters 3, 4, 6 and 7. The data on the ovary in situ, the utero-ovarian transplant and the effects of prostaglandin $F_{2\alpha}$ are from experiments by Dr. D.T. Baird in which I measured the plasma progesterone concentrations.

Parts of this project have been published with the consent of my supervisors ( Baird, Collett & Land, 1971; Collett, Land & Baird, 1973; Baird & Collett, 1973 ).

1st. July 1973

ABSTRACT

A long term surgical preparation in which the sheep ovary is autotransplanted to a superficial site with vascular reanastomoses was used to study ovarian function. The changes in progesterone secretion rate were followed during close intra-arterial infusion of substances into the ovary.

A competitive protein binding method for measuring progesterone concentrations in sheep plasma was developed. The specificity, accuracy and precision of this assay were judged to be satisfactory for the purposes of this study.

The effect of infusing saturating doses of luteinizing hormone was studied. Luteinizing hormone produces only a transient rise in secretion rate, and the pattern of this response was characterized. Human chorionic gonadotrophin had a similar effect. After previous trophic hormone stimulation, the ovary becomes refractory to further stimulation for at least two hours. Two experiments with prolactin were inconclusive.

The luteolytic action of prostaglandin $F_{2\alpha}$ *in vivo* was confirmed. Prostaglandin infusion was used as a method to cause the formation of a new *corpus luteum* after ovulation at the ensuing oestrus. The sensitivity of *corpora lutea* to luteinizing hormone was found to vary with the age of the luteal tissue and previous hormone treatment.

Aminophylline, cyclic-3',5'-adenosine monophosphoric acid, and a more active derivative were infused into the ovary, but none of these substances increased progesterone secretion.

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The experimental work was performed while I was employed in a technical capacity: firstly in the Department of Obstetrics and Gynaecology, University of Edinburgh; and then by the Medical Research Council, Unit of Reproductive Biology. Much of the thesis was written while I held a demonstratorship in the Department of Veterinary Pharmacology, Royal (Dick) School of Veterinary Studies, University of Edinburgh. I am grateful to my three heads of department; Prof. R.J. Kellar, Dr. R.V. Short and Prof. F. Alexander for the support and facilities that they made available to me.

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Previously published work is acknowledged by means of references which are cited in full in an appendix.

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ABBREVIATIONS AND TRIVIAL NAMES

## Steroids

| | |
|---|---|
| adrenosterone | 4-androstene-3,11,17-trione |
| cortisol | 11$\beta$,17,21-trihydroxy-4-pregnene-3,20-dione |
| pregnenolone | 3$\beta$-hydroxy-5-pregnen-20-one |
| progesterone | 4-pregnene-3,20-dione |
| 20$\alpha$-dihydroprogesterone | 20$\alpha$-hydroxy-4-pregnen-3-one |
| 17$\alpha$-hydroxyprogesterone | 17$\alpha$-hydroxy-4-pregnene-3,20-dione |
| oestradiol-17$\beta$ | 1,3,5,(10)-estratriene-3,16$\alpha$,17$\beta$-triol |
| oestrone | 3-hydroxy-1,3,5(10)-estratrien -17-one |

## Hormones

| | |
|---|---|
| ACTH | adrenocorticotrophin |
| FSH | follicle stimulating hormone |
| GH | growth hormone |
| HCG | human chorionic gonadotrophin |
| LH | luteinizing hormone |
| PMSG | pregnant mare serum gonadotrophin |
| TSH | thyroid stimulating hormone |

CHAPTER ONE

# INTRODUCTION

Stresses caused by a rapidly expanding world population have focussed attention onto the biology of reproduction. Great strides have already been taken to alter the natural fertility of man and farm animals. Nevertheless, it is clear that unless current programmes designed to increase animal fertility and to decrease the growth of the human population are intensified, then even less food will be available in poor countries. Moreover, the quality of life in advanced countries is likely to suffer further because aggressive and disruptive acts appear to be a characteristic response to overcrowded living conditions.

Exceedingly effective and well tolerated methods of preventing conception in man are now available, and it has been suggested that legal and other obstacles are likely to make the introduction of an entirely new antifertility drug uneconomic. Furthermore, the adoption of even the least satisfactory method of avoiding conception in India would have a more dramatic effect on that country's rate of population growth than any of the existing family planning programmes have yet achieved ( Potts, 1972 ). It would appear that the major problems in reducing the human population growth are economic, social and religious.

Methods of increasing fertility are inadequate and progress in the practical field is likely to be made only when there is a better understanding of the controlling mechanisms in reproductive biology ( Rowson, 1971 ). In medical practice, a small number of couples present at infertility clinics. Although gonadotrophin therapy has proved beneficial ( Gemzell, 1965 ), the incidence of multiple pregnancies is disturbing.

During the last decade, ultra-sensitive and rapid methods have been developed for assaying hormones. Progress in experimental surgery has also been fast. At the time that this project was started ( 1969 ), it was considered that the application of these new techniques could yield new information about the *in vivo* responses of the ovary towards gonadotrophins and other materials.

## Historical aspects

An outline of early developments in reproductive biology has been provided by Asdell ( 1969 ). Theories about the rôle of the pituitary, the gonads and the uterus in the cyclical phenomena of reproduction were formulated at the turn of the century. The writings of Heape and Marshall have proved to be the most enduring. Heape carefully defined the stages of the sexual cycle. Marshall noted a close association between the prominent ephemeral ovarian structures - *corpora lutea* and large follicles - and certain conditions of the reproductive tract. The concept of the ovary as a gland of internal secretion soon became established.

A large number of observations indicated that changes in the appearance of the gonads, especially those occuring at puberty, were determined by a 'generative ferment' produced outside the gonads, possibly by the pituitary. The phenomena of compensatory hypertrophy after unilateral castration, the 'law' of follicular constancy and the 'unbolting' effect could all be explained by that theory. These early experiments have been outlined by Parkes ( 1966 ).

The internal secretions of the ovary were characterized during the thirties. An active substance in corpora lutea causing endometrial proliferation, suppressing ovulation and which maintained pregnancy in spayed animals was identified as a steroid and named progesterone ( see Allen, 1939 ). Oestrogens were also isolated; they were found to induce oestrus and they produced a typical reaction in the uterus, the vagina and the mammary gland ( see Doisy, 1939 ).

Work on the anterior pituitary secretions was less rapid because ablation of the pituitary is difficult. In the thirties it was recognized that there were two hormones which had effects on the gonads. Luteinizing hormone ( LH ), otherwise known as interstitial cell stimulating hormone, was found to be responsible for ovulation in the female and testosterone secretion in the male. Follicle stimulating hormone ( FSH ) was responsible for follicular growth in the female and it maintained testicular tubules in hypophysectomized males. This early work has been reviewed ( Fevold, 1939 ).

Luteotrophic hormones

Further work on extracts of anterior pituitaries revealed that there was a third hormone concerned with reproduction. This hormone was named prolactin because it promoted milk secretion. Although prolactin was purified before LH and FSH, its rôle in the regulation of the ovary was discovered later. In rats, prolactin was found to increase progesterone secretion, to prolong the life span of the corpus luteum and to maintain these structures in hypophysectomized animals ( Astwood, 1953 ). Luteotrophic activity was distinguished from that which induced ovulation.

However, the corpora lutea of rodents are atypical because they remain non-functional after ovulation unless a further stimulus is provided. The corpora lutea of ewes, cows and sows become functional after ovulation without a further stimulus, and their life span is ended by a uterine factor in much the same way as pseudopregnancy is ended in the rat ( see Anderson, Bland & Melampy, 1969 ). These differences make prolongation of luteal function an unsatisfactory test for luteotrophin in farm animals ( Everett, 1961 ): in them, and in primates, a positive stimulus from the pregnant womb is required to prevent luteal regression ( Short, 1964 ).

The effects of hypophysectomy, and the ability of hormone preparations to restore the activity of the gonads of hypophysectomized animals have been reviewed ( Rowlands & Parkes, 1966 ). An unexpected finding was that prolactin and LH had a luteolytic action on the non-functional corpora lutea of chronically hypophysectomized

rats. These paradoxical effects were reviewed by Rothschild ( 1966 ) who suggested that no single hormone would be able to maintain the corpus luteum of hypophysectomized animals. It was proposed that a 'luteotrophic complex' consisting of several hormones was necessary, and that the relative importance of particular hormones varied from species to species.

Stimulation of progesterone synthesis either in vitro or or in vivo has been suggested as a suitable test for luteotrophic activity. However, it is possible that luteal tissue from some species is already secreting progesterone at a maximum rate so that further gonadotrophic stimulation would prove ineffectual ( Short, 1964 ). Within the last few years, antibodies to hormones and pituitary blocking agents have been used to investigate the degree of pituitary support required by the gonads.

Studies in vitro

A very frequently used method for studying ovarian metabolism is to incubate tissue minces or slices with precursors for a few hours and then stop the reactions and analyse the products formed.

The use of in vitro incubations and radioactive precursors has enabled the biosynthetic pathways for ovarian steroids to be formulated ( Ryan & Smith, 1965 ). Furthermore, the different metabolic capabilities of different cells have been identified; granulosa cells and their derivatives were found to make progesterone

whereas theca cells and their derivatives make oestrogens ( Short, 1964 ).

The effect of adding LH - but not FSH or prolactin - to *in vitro* incubations of luteal tissue was to increase the amount of progesterone synthesized ( Savard, Marsh & Rice, 1965; Armstrong, 1968 ). Although artefacts are caused by enzymes which leak from damaged cells in tissue slices, and by cell walls which act as permeability barriers, the major site of action of LH is now thought to be between cholesterol and pregnenolone ( Armstrong, 1968; Savard, Le Maire & Kumari, 1969 ). Cyclic-3',5'-adenosine monophosphoric acid (cAMP) is believed to act as a 'second messenger' in mediating the steroidogenic effect of luteinizing hormone ( Marsh, Butcher, Savard & Sutherland, 1966 ), because exogenous cAMP mimics the effect of LH. Besides stimulating steroidogenesis, LH also increases ovarian glucose metabolism, stimulates ovarian cholesterol esterase and decreases ovarian cholesterol ester synthesis. No doubt these changes are responsible for the cholesterol depleting activity which LH shows *in vivo*. Prolactin was found to have effects opposite to those of LH on cholesterol ester metabolism.

The immature rat ovary has also been studied by *in vitro* incubation. Unlike luteal tissue, it appears to respond to both LH and FSH. The granulosa cells respond to LH and the theca cells to FSH by showing an increased rate of glycolysis ( Ahrén, Hamberger & Rubenstein, 1969 ). Changes in nucleic acid and protein metabolism also occur ( McKerns, 1969 ).

Incubation studies destroy the cellular relationships which exist in the original organ, and products of metabolism accumulate in the medium. Both these objections may be overcome in the technique of organ perfusion. Bartosik and Romanoff ( 1969 ) perfused bovine ovaries: they showed LH and prolactin to stimulate progesterone secretion and that cholesterol was taken up from the perfusing medium.

Long term studies *in vitro* have been carried out by culturing granulosa cells ( Channing, 1970 ). Cells from preovulatory follicles were found to luteinize spontaneously, whereas cells from small follicles required LH and FSH for this transformation. Cyclic AMP was able to mimic the effects of gonadotrophins. However, abnormal cytological changes frequently seen in cultured lines of cells make the results of such experiments difficult to interpret.

None of the *in vitro* techniques can substitute for the conditions existing *in vivo*. In addition to the qualifications already mentioned, *in vitro* systems necessarily lack the influences provided by the nervous and vascular systems.

Studies *in vivo*

Few attempts have been made to verify ovarian biosynthetic pathways *in vivo* and these have been reviewed ( Aakvaag & Eik-Nes, 1969 ). In an ingenious experiment, tritiated pregnenolone was injected into the follicular fluid of a mare ( Short, 1964 ). No conversion into tritiated oestrogen was detected, thus confirming *in vitro* experiments.

The action of gonadotrophins on ovarian steroid secretion has been studied by several methods. In indirect methods, the secretion rate of hormone is inferred from the rate of excretion of urinary metabolites or from the concentration of hormone in peripheral plasma. These methods are not fully satisfactory. If gonadotrophins are administered systemically, the observed effects may be mediated by other organs, especially the pituitary. The analysis of urine has been used to follow the effect of gonadotrophin injections in women ( Gemzell, 1965 ). However, urinary results may be influenced by the liver, the kidney or, in the case of steroids exhibiting enterohepatic circulation, by the contents of the intestine. If the metabolite being analysed is produced by other glands, especially the adrenal, then this too will have to be taken into account in the interpretation of results. Finally, urinary excretion is only able to give an indication of the mean secretion rate over a long period of time. Similar difficulties arise when peripheral hormone concentrations are measured. For example; Schomberg, Coudert and Short ( 1967 ) showed LH to induce a brief elevation in peripheral progesterone concentration in heifers. Yet, they were not able to exclude an increase in adrenal secretion rate or a decrease in the metabolic clearance rate of progesterone as being responsible for their results.

It is concluded that unequivocal estimates of secretion rates may be obtained only when blood flow through the gland is measured and when the differences between arterial and venous concentrations of the secreted hormone are known. Often, however, the concentration of hormone in peripheral plasma is taken to be the same as that in arterial plasma.

The ovarian vein must be cannulated if ovarian effluent blood is to be collected. Consequently, most studies of the ovary in vivo have been made at operation under acute conditions of anaesthesia. Previous work in this field has been reviewed critically ( McCracken & Baird, 1969 ). They concluded that previous experiments had left much to be desired: during operation the abdominal viscera are handled ; not all investigators had taken proper steps to ensure complete collection of the effluent ovarian blood; mostly no attempt had been made to measure ovarian blood flow; and finally, the effect of gonadotrophins could have been masked by circulating anaesthetic drugs. The last objection was verified in a study where pentobarbital was used ( Hixon & Clegg, 1969 ). No anaesthesia is required for the collection of ovarian vein blood from animals bearing a chronically implanted ovarian vein catheter. However, in such a preparation, it is difficult to keep the catheter patent ( Lindner, Sass & Morris, 1964 ). Unless the ovarian artery is also catheterized ( for example, Thorburn & Nicol, 1971 ), it is not possible to infuse substances directly into the ovary. In none of these preparations is it possible to conduct long term studies.

All the above disadvantages are overcome in a new long term surgical preparation where the sheep ovary is transplanted to a superficial site in a skin fold in the neck, and where the major blood vessels are reanastomosed ( Goding, McCracken & Baird, 1967 ). Studies conducted with this preparation before the start of this project have been reviewed ( McCracken & Baird, 1969 ). Assessment of the transplanted gland showed that the histology was normal, that the major ovarian steroids were secreted, and that the ovary would respond to gonadotrophin. Only in one respect was the transplanted

ovary abnormal; the corpora lutea fail to regress, presumably because luteal regression is controlled by the uterus in sheep ( Short, 1964 ). The transplanted ovary will have had its nervous supply interrupted, but even in situ, the ovary appears to be poorly innervated. It was thought that this preparation was ideal for studying the acute effects of gonadotrophins in conscious animals. Since the start of this project, a further paper assessing the function of this preparation has appeared ( Goding, Baird, Cumming & McCracken, 1972 ).

A slightly different preparation has been described, again using sheep, where the ovary has been allowed to remain in situ, but the ovarian vein has been anastomosed to the mammary vein ( Thorburn & Mattner, 1971 ). Although access to the arterial circulation is not provided, it is possible to sample ovarian vein blood in the conscious ewe. Retrograde blood flow with consequent dilution of the ovarian effluent is difficult to exclude.

Sheep as experimental animals

Besides being of great economic importance, sheep have established themselves in the last few years as a significant laboratory species in their own right. There are several good reasons for this trend. They are relatively cheap to buy, to feed and to house. Sheep, ewes especially, are docile, they do not bite, and they can be trained to cooperate while experiments are taking place. For this project, ewes are particularly desirable on account of their size. They are neither so big nor so small as to require specialized surgical skill or special instruments at operation. Ewes can easily withstand the

withdrawal of 200 ml blood without ill-effects. The facility of obtaining large volumes of plasma was for a long time essential if precise steroid analyses were to be carried out. Although this requirement is no longer essential, less exacting assay systems than would otherwise be needed, are fully satisfactory in this species.

Outline of the reproductive endocrinology of the non-pregnant ewe

Oestrus and ovulation

The ewe is a seasonal, polyoestrous breeder. During the winter months she comes into heat at regular time intervals of 17 to 18 days for a period of 24 to 36 hours. Only when she is in heat will she allow the ram to mount her and mate. The length of the breeding season is breed dependent, but it is said to be extended by a plentiful supply of food. Ovulation is spontaneous. These facts were verified and reviewed at the turn of the century ( Marshall, 1903 ). Robertson ( 1967 ) and McKenzie and Terrill ( 1937 ) found ovulation to occur 24 to 30 hours after the onset of heat.

Marshall, and later workers, showed that pro-oestral changes in the uterus - when much mucus is discharged - were correlated with the presence of a large follicle, and that these took place just before the onset of heat ( Marshall, 1903; Grant, 1934; McKenzie & Terrill, 1937 ). Similar changes in the uterus and the ovaries could sometimes be observed without the accompaniment of behavioural oestrus ( Grant, 1933 ). Such 'silent' ovulations were most typically seen before the start of the breeding season. Recently, waves of follicular growth and atresia have been described during dioestrus ( Brand, 1971; Smeaton & Robertson, 1971 ).

The corpus luteum

Formation of the corpus luteum from granulosa cells from the ovulated follicle has been described ( Marshall, 1903; Warbritton, 1934; Grant, 1934 ). By day 8 of the cycle - the day of onset of heat is designated day 0 - the corpus luteum is fully functional, and it has reached its maximum size and weight ( Edgar & Ronaldson, 1958; Deane, Hay, Moor, Rowson & Short, 1966 ). Regression of the gland is very rapid, and takes place on about day 15, although some ultrastructural changes may be detected as early as day 13 ( Deane et al., 1966 ).

The corpus luteum is necessary for the maintainance of pregnancy during the first trimester ( Casida & Warwick, 1945 ), but progesterone injections will save a ewe spayed early in pregnancy from abortion ( Foote, Gooch, Pope & Casida, 1957 ).

Effect of exogenous hormones

A fall in the level of progesterone is required before the spayed ewe will show heat after the injection of a small amount of oestrogen ( Robinson, 1954 ). Administration of progestagens prevents ovulation, and they may be used for synchronizing oestrus ( Robinson, 1967 ).

During the summer months when the ewe is in anoestrus, ovulation and heat may be induced by injections of gonadotrophins or oestrogen ( Cole & Miller, 1935; Hammond, Hammond & Parkes, 1942 ).

Control of luteal life span

In sheep, the uterus controls luteal regression. Hysterectomy results in luteal maintenance ( Wiltbank & Casida, 1956 ). In a series of elegant experiments, it has been shown that the luteolytic effect is exerted locally and that it can be blocked by pregnancy ( Moor & Rowson, 1968 ). Corpora lutea of hysterectomized

animals regress slowly contrasting with the abrupt decline seen at the end of the oestrous cycle ( Short, 1964 ).

The effects of hypophysectomy and stalk section on luteal function have been described ( Short, 1964; Denamur, Martinet & Short, 1966 ). If hypophysectomy is carried out just after ovulation, a new corpus luteum forms, but its functional life is shorter than normal. Hypophysectomy during mid-cycle results in regression, as does hypophysectomy of a hysterectomized ewe bearing a maintained corpus luteum. After stalk section, some prolactin secretion continues, and the effects on luteal function are less severe than after hypophysectomy.

Hormone levels

At the start of this project ( 1969 ), there were few reliable estimations of the concentrations of sex hormones - apart from progesterone - in sheep plasma. Since then, there has been an explosion in the available information. Little of this recent work would have been possible without the use of radioimmunoassay systems.

Reliable chemical methods for measuring progesterone concentrations were first used for ovarian vein plasma. Very low levels were found at oestrus; a plateau was observed between days 8 and 13; and concentrations fell rapidly on day 14 or 15 ( Edgar & Ronaldson, 1958; Short, 1964; Moore, Barrett, Brown, Schindler, Smith & Smyth, 1969 ). Progesterone secretion rates followed the same pattern. It was concluded that secretion represented synthesis rather than release because the progesterone content of corpora lutea was small in relation to the rate of secretion ( Stormshak,

Inskeep, Lynn, Pope & Casida, 1963; Short, McDonald & Rowson, 1963 ). Peripheral progesterone concentrations follow a pattern similar to that seen in the ovarian vein ( Stabenfeldt, Holt & Ewing, 1969; Thorburn, Bassett & Smith, 1969 ). The rate of metabolism of progesterone in blood has been determined, and the half-life found to be a few minutes ( Short & Rowell, 1962 ).

Pituitary FSH and LH contents were found to rise during dioestrus. The gonadotrophins are released just after or before the onset of oestrus. LH release and ovulation may be prevented by injections of tranqillizer given just before the expected time of oestrus ( Robertson, 1967 ).

Plasma LH concentrations are very low during most of the cycle except for a brief peak at the onset of oestrus. LH discharged from the pituitary is metabolized with a half-life of about 20 minutes ( Geschwind & Dewey, 1968 ). Injections of oestrogen during anoestrus ( Goding, Catt, Brown, Kaltenbach, Cumming & Mole, 1969 ), or of LH releasing hormone ( Reeves, Arimura, Schally, Kragt, Beck & Casey, 1972 ) induced LH discharge from the pituitary.

Prolactin is released episodically and it has a short half-life in blood: consequently, wide variations in concentrations between individual samples are observed. Peak concentrations are generally seen 3 to 18 hours after the onset of oestrus and 1½ to 10 hours after the highest LH concentrations ( Cumming, Brown, Goding, Bryant & Greenwood, 1972 ).

Oestrogen concentrations in peripheral plasma are likely to be very low. However, they have been measured in ovarian vein plasma ( Moore et al., 1969; Scaramuzzi, Caldwell & Moor, 1970 ). The ovary secretes much more oestradiol-17$\beta$ than oestrone, and the highest secretion rates are seen from ovaries bearing preovulatory follicles. Minor peaks are also seen during dioestrus and they have been correlated with waves of follicular growth leading to atresia ( Cox, Mattner & Thorburn, 1971 ).

The time relationships between the onset of oestrus and the peak levels of FSH, LH, prolactin, and oestrogen have been determined ( Bjersing, Hay, Kann, Moor, Naftolin, Scaramuzzi, Short & Younglai, 1972 ).

Luteolysis

The rapid regression of corpora lutea on day 14 has been noted already. A natural luteolytic hormone has been identified as prostaglandin $F_{2\alpha}$. Concentrations of this substance in the uterine vein are higher at the end than early in the oestrous cycle ( Bland, Horton & Poyser, 1971; McCracken, Carlson, Glew, Goding, Baird, Green & Samuelsson, 1972 ).

A number of procedures cause luteolysis such as: administration of progesterone during the early part of the cycle ( Woody, First & Pope, 1967 ), fitting intrauterine devices ( Moore & Nalbandov, 1953; Ginther, Pope & Casida, 1966 ), and administration of oestrogen at mid-cycle ( Akbar, Rowe & Stormshak, 1971 ). The effects of progesterone and oestrogen appear to be mediated by the uterus, and it is tempting to speculate that the synthesis and

release of prostaglandin $F_{2\alpha}$ is controlled by the action of these steroids on the uterus. It is possible that irritation causes prostaglandin release, and the effects of steroids already noted could account for their ability to enhance or suppress the action of intrauterine devices.

CHAPTER TWO

# MATERIALS AND METHODS

In this chapter, the methods used throughout these studies are described. Accounts of methods peculiar to specific experiments are to be found in the materials and methods sections of the appropriate chapters. This chapter comprises four main parts describing: a competitive protein binding assay for progesterone and its development; the experimental ewes; the statistical analysis of results; and some results of investigations that allow an assessment of the function of the transplanted ovary.

## THE PROGESTERONE ASSAY

### A review of previously published methods

Early work leading to the purification of the active pregnancy hormone from corpora lutea, and to the identification of progesterone has been reviewed ( Allen, 1939 ). Those advances were made possible by the use of bioassays based on the reactions of uteri of test animals towards injected extracts. Bioassay methods for progesterone have been reviewed ( Miyake, 1962 ). In general, these assays are insensitive, not very specific, they require many

animals, and they require considerable time. Except for measuring the progestogenic activity of synthetic compounds, these methods are obsolete. However, statistical methods were developed to analyse the results of bioassays ( Emmens, 1962 ), and these ideas have been applied to the data resulting from modern immunoassay systems. The so-called 'radioreceptor' assays may be regarded as vastly simplified in vitro bioassays.

At first, chemical estimation of progesterone depended on the absorption of light at 240 nm, a characteristic of 4-en-3-one steroids. Other steroids that absorb light of this wavelength have to be removed by chromatography. Although the formation of coloured derivatives has been proposed, none of those methods appears to offer advantages ( Short, 1961 ). Spectrophotometric methods require 0.5 µg for a satisfactory estimation ( Short, 1961; Zander, 1962 ). When a chemical method was compared with a sensitive bioassay, the latter method gave the higher results, and it was concluded that the chemical method was more specific ( Edgar, Flux & Ronaldson, 1959 ).

During the decade 1960-1970 there was a rapid advance in analytical technique. Assays capable of detecting nanogramme amounts of progesterone were published. The availability of high specific activity radioactive steroids, and of high performance electronic equipment, made these advances possible. Radioactively labelled steroids enabled workers to correct for the procedural losses involved in chromatographic steps, and the new apparatus allowed detection systems more sensitive than spectrophotometry to be used; for example; fluorimetry ( Heap , 1964 ), and flame ionization detection ( Deane,

Hay, Moor, Rowson & Short, 1966 ). The technique of liquid scintillation counting made possible ultra-sensitive double isotope derivative methods ( Riondel, Tait, Tait, Gut & Little, 1965) and the displacement methods now being used for steroid analysis.

Methods for progesterone based upon gas liquid chromatography have been reviewed ( van der Molen & Aakvaag, 1967 ). Electron capture detection of chloroacetate derivatives of progesterone has allowed the detection of as little as 1 ng progesterone ( Stabenfeldt, Ewing, Patton & McDonald, 1969 ).

Work by Yalow and Berson showed that patients treated with bovine insulin possessed antibodies to that hormone in their blood. Radioactively labelled insulin bound to their plasma proteins in vitro. Yalow and Berson realized that the fraction of labelled insulin bound to antibody was a quantitative function of the total insulin concentration, and they developed an assay based on this principle ( see Yalow & Berson, 1971 ).

The fundamental equation describing the behaviour of this system is:

$$\begin{array}{ccc} \text{ANTIGEN} & & \text{ANTIGEN-ANTIBODY} \\ + & \text{ANTIBODY} \rightleftharpoons & + \\ \text{LABELLED-ANTIGEN} & & \text{LABELLED-ANTIGEN-ANTIBODY} \end{array}$$

The presence of antigen in samples is indicated by displacement of labelled antigen off the antibody. The amount of

antigen in samples is estimated by comparing the degree of competitive inhibition observed with that produced by adding known amounts of antigen. The validity of this type of assay depends on identical behaviour of hormone in unknown and standard solutions, whereas isotope dilution methods require identical behaviour of labelled and unlabelled material.

It was soon realized that any polypeptide hormone could be measured by this principle once suitable antisera had been prepared. This type of assay is known as radioimmunoassay ( RIA ). Other proteins bind molecules of great physiological interest, and such proteins have been used in competitive protein binding assays ( CPB ) for thyroxine, vitamine $B_{12}$ and in particular, corticosterone binding globulin has been used for CPB assays for adrenocorticosteroids and progesterone ( Murphy, 1969 ).

The main purpose of the progesterone assay in this project was to follow variations in the concentration of this hormone in sheep ovarian vein plasma. However, it was also desirable to be able to measure peripheral plasma concentrations of this hormone. Apart from the sensitivity required, it was necessary to have a method that could cope with 50 samples in a fortnight.

Many modern assays have the required degree of sensitivity, but they are far too time consuming ( Riondel et al., 1965; Stabenfeldt et al., 1969 ). Although previous studies on the sheep ovarian autotransplant had used a gas liquid chromatography method for progesterone ( McCracken, Uno, Goding, Ichikawa & Baird, 1969 ), it was

decided to develop a CPB method with, it was hoped, greater rapidity and sufficient sensitivity to enable peripheral plasma progesterone to be measured.

This project was started in 1969, and at that time two reports of CPB methods for progesterone had been published ( Neill, Johansson, Datta & Knobil, 1967; Yoshimi & Lipsett, 1968 ). Shortly afterwards, a simpler method suitable for sheep peripheral plasma samples was reported ( Thorburn, Bassett & Smith, 1969 ). The following pages describe a method which I developed from these three reports. The method was tested critically, and the results are discussed.

Materials

Solvents - The following organic solvents were obtained from British Drug Houses ( Poole, Dorset ): petroleum ether ( boiling point 40 - 60°C, and boiling point 100 - 120°C ) 'Analar' grade; toluene, 'Analar' grade; ethyl acetate 'Analar' grade; chloroform, 'Analar' grade; diethyl ether, 'Microanalytical' grade; absolute ethanol, 'Aristar' grade; methanol 'Aristar' grade; cyclo-hexane ( special for spectroscopy ); and n-hexane ( boiling point 67 - 70°C, free from aromatic hydrocarbons ). Where it was necessary to redistil solvents, glass apparatus ( 'Quickfit & Quartz', J.A. Jobling, Stone, Staffordshire ) was used. Distilled water was obtained from an electrically heated still in the laboratory ( Manesty Machines, Liverpool ).

Steroids - The following steroids were used: progesterone ( 4 -pregnene-3,20-dione ) from Steraloids, Croydon, Surrey; 20α-dihydroprogesterone ( 20α-hydroxy-4-pregnen-3-one ) gift, Dr. D.T. Baird; cortisol ( 11β,17,21-trihydroxy-4-pregnene-3,20-dione ) gift from Dr. J. Seth, department of Clinical Chemistry, Royal Infirmary, Edinburgh; 17α-hydroxyprogesterone ( 17α-hydroxy-4-pregnene-3,20-dione ) from Sigma, Kingston, Surrey; pregnenolone ( 3β-hydroxy-5-pregnen-20-one ) from Steraloids, batch 8050; and adrenosterone ( 4 -androstene-3,11,17-trione ) from Sigma, lot 598-0250.

Radioactively labelled steroids - These were obtained from the Radiochemical Centre ( Amersham, Buckinghamshire ) as follows: 1α,2α-$^{3}$H-progesterone ( batch B1, 30 Ci/mM and batch B5, 53 Ci/mM ); 7α-$^{3}$H-17α-hydroxyprogesterone ( batch B2, 10 Ci/mM ); 7α-$^{3}$H-pregnenolone ( batch B4(RT), 5 Ci/mM ) and 4-$^{14}$C-pregnenolone ( batch B10, 50 mCi/mM ). 7α-$^{3}$H-cortisol ( 30 Ci/mM ) was a gift from Dr. J. Seth. 1α,2α-$^{3}$H-progesterone was also obtained from New England Nuclear ( Boston, Massachusetts, U.S.A. ) batch 321-124, 55 Ci/mM. The steroids were stored at 4°C in ethanolic solution. They were purified by paper chromatography before use.

Automatic pipettes - Volumes smaller than 100 µl were dispensed by means of a Hamilton syringe with a Chaney adaptor ( Micromesure, The Hague, Netherlands ). Volumes between 0.1 and 1.0 ml pipetted by means of the 'Selecta-pipette' ( Becton, Dickinson ( U.K.), Wembley, Middlesex ). Petrol ether was delivered by a 'D-L' syringe ( Aimer Products, London ).

Phosphate buffer - For CPB assay a pH 7.4 phosphate buffer was made up as follows: 7.62 g/l anhydrous disodium hydrogen orthophosphate, 1.78 g/l potassium dihydrogen orthophosphate and 0.5 g/l sodium azide. The first two salts were 'Analar' grade, the third technical grade and all three salts were obtained from British Drug Houses.

Binding protein - Third trimester, human, pregnancy blood was obtained by venupuncture from patients attending antenatal clinics at the Simpson Maternity Pavilion by Dr. D.T. Baird. The blood was heparinized ( Weddel Pharmaceutical, London ) and the red cells were removed by centrifugation at 4°C. The plasma was incubated with 50 mg/ml powdered charcoal ( Norit A, British Drug Houses ) at room temperature for 30 minutes in order to absorb endogenous steroids. The plasma was centrifuged at 500 g for 20 minutes and the supernatant was withdrawn and filtered over Whatman No. 1 paper. Aliquots of plasma ( 1 ml ) were deep frozen until required.

Assay reagent - Binding protein solution for the assay was prepared by thawing an aliquot and diluting it. For measuring ovarian vein samples 1 ml was diluted to 20 ml with phosphate buffer. For measuring peripheral plasma samples 1 ml was diluted to 50 ml with buffer and 0.1 % gelatin ( Sigma ) was added in order to minimize the adsorption of binding protein onto glass surfaces. An ethanolic solution of tritiated progesterone containing $4 \times 10^6$ d.p.m. ( about 1 ng mass ) was placed at the bottom of a test tube and the solvent evaporated before the addition of dilute plasma solutions. The mixture was incubated for 15 minutes at 37°C. Such solutions could be stored at 4°C for up to two weeks without deterioration.

Glassware - 'Pyrex' and 'Quickfit & Quartz' glassware ( J.A. Jobling ) was used. After use, it was soaked in a solution of 'Pyroneg' ( Diversey, Barnet, Hertfordshire ), rinsed in tap water, and then soaked in 5 % 'Decon 90' ( Decon Laboratories, Brighton, Sussex ). When the glassware had been removed from the 'Decon' solution, it was rinsed repeatedly with tap water and then with distilled water. Glassware was dried in a hot air oven.

Methods

Determination of radioactivity - Scintillation fluid was a solution of 42 mg/l 2,5-diphenyloxazole and 5.3 mg/l 1,4-bis-2-(5-phenyloxazolyl)-benzene in toluene containing 1 % v/v absolute ethanol. Both fluors were from Packard ( Wembley, Middlesex ). Radioactive samples were placed in low background, glass scintillation phials ( Packard ). Samples dissolved in organic solvents were treated by evaporating the solvent, and 10 ml scintillation fluid was added. Aqueous samples containing tritiated progesterone were counted after the sample had been mixed vigorously with 10 ml scintillation fluid. A model 3320 Packard liquid scintillation spectrometer with the cabinet temperature set at 6°C was used for counting samples. The instrument settings were: 50-1000 channel width, 50 % gain for tritium, and 50-1000 channel width, 8.4 % gain for carbon-14. The efficiency of counting was 49 % for tritium and 88 % for carbon-14. The background count rate was 27 c.p.m. for the tritium channel and 13 c.p.m. for the carbon-14 channel. No correction was made for quenching because there were no significant differences between the counting efficiencies of individual samples.

Paper chromatography - Paper chromatography of steroids was performed with the precautions advised by Bush ( 1961 ). Descending chromatography was done on 60 × 3 cm strips ( Whatman No.1, H. Reeves Angel, Croydon, Surrey ) which had been extensively washed by descending methanol. Steroids were located by the use of an ultraviolet lamp ( 254 nm, Camlab, type TL900 ) and radioactivity was detected with a Packard strip scanner. Steroids were eluted with 10 ml ethanol. The system ligroin 100: methanol 85: water 15 was used for progesterone and toluene 100: methanol 75: water 25 for cortisol ( Bush, 1961 ).

Thin layer chromatography (TLC) - TLC was performed as described previously ( Baird, Goding, Ichikawa & McCracken, 1968 ). It was used for purifying progesterone from plasma extracts.

Alumina column chromatography - Progesterone spots were eluted from TLC plates and subjected to alumina chromatography ( Yoshimi & Lipsett, 1968 ). Aluminium oxide was grade 2 ( Brockmann & Schodder, 1941 ). It was washed with 6 N hydrochloric acid, rinsed with water until neutral, with methanol and finally methylene dichloride. It was activated by incubation at 60°C in an oven overnight.

Gas liquid chromatography (GLC) - Analyses were carried out using a Pye 104 series machine. A flame ionization detector was linked to a chart recorder. A three foot long glass column was packed with 'Gas ChromQ' ( Field Instruments, Richmond, Surrey ). ½ % OV-1 was used as the liquid phase ( Phase Separation,

Queensferry, Flintshire ). After conditioning the column at 230°C for 2 days, the column was maintained at 210°C. The carrier gas, argon, was supplied at a flow rate of 40 ml/min. For the detector system, air was supplied at 12 p.s.i. and the hydrogen gas flow rate was 80 ml/min. The attenuation setting for the detector was $5 \times 10^{-10}$ . Dried extracts of standard and unknown samples were dissolved in 100 µl ethanol containing 2 µg adrenosterone. The solution was mixed and a 10 µl aliquot removed for counting in order that the results might be corrected for procedural losses of extraction and purification. The solvent in the remaining part of the sample was evaporated, and the residue taken up in 5 µl ethanol. Two 2 µl injections were made into the GLC column by means of a Hamilton syringe. A plot of the ratio of the adrenosterone and progesterone peak heights against amount of added progesterone was linear over the range 0 to 2 µg progesterone per sample. A calibration curve was determined on each day that unknown samples were run.

Known amounts of progesterone were added to peripheral plasma taken from a castrate ewe to give concentrations in the range of 0 to 1 µg/ml. When 4 ml samples were analysed after TLC and alumina column chromatography, the estimates were very near the concentrations added. The regression equation for the amount estimated, y, was: $y = ( 1.14 \pm 0.08 ) x - ( 0.01 \pm 0.07)$, where 'x' was the concentration of progesterone added ( n = 10 ).

Petrol ether extraction - Plasma samples ( 1 ml ) were extracted by shaking them twice with 4 ml petroleum ether ( boiling point 40 - 60°C ). The combined extracts were washed with water,

and the organic phase was transferred to a fresh tube ( Thorburn, Bassett & Smith, 1969 ). Petrol ether was evaporated under a stream of filtered nitrogen gas in a water bath at 45°C. The dried extract was dissolved in 2 ml absolute ethanol. Suitable aliquots were removed to assay tubes ( 3 ml conical tubes ), and the ethanol was driven off by incubating the tubes in a vacuum oven ( Townson & Mercer, Croydon, Surrey ) at 55°C. The vacuum was generated by a 'Speedivac' pump ( Edwards, Crawley, Essex ). In some experiments, 1,000 c.p.m. tritiated progesterone was equilibrated with each sheep plasma sample, and the procedural losses incurred by this method were determined. The recovery of radioactivity was 90.5 ± 0.50 ( S.E.M. ) %; n = 27. This step was later omitted because the recovery was both high and very reproducible.

The specificity of the extraction step was assessed by following the recovery of radioactivity from samples of ovine plasma to which tritiated cortisol or tritiated 17α-hydroxyprogesterone had been added. Less than 0.05 % of the radioactivity from cortisol was recovered in the combined petrol ether extracts. Of the radioactivity from 17α-hydroxyprogesterone, 35 % was recovered, but this was lowered to 25 % of the original amount after washing the petrol ether extract with water. In a study on the petrol ether extracts from samples of experiment 3, the extracts were purified by TLC and alumina column chromatography. 69.8 ± 2.35 ( S.E.M. ) % of the radioactivity of added tritiated progesterone was recovered ( n = 24 ).

Incubation - Assay reagent ( 0.1 ml binding protein solution with tritiated progesterone ) was added to 3 ml assay tubes to

which standard or unknown amounts of progesterone had been added. The mixture was shaken gently and incubated at 37°C for 15 minutes, and then for 2 hours at 0°C. Extending the warm incubation period to 1 hour, or the cold incubation period to 12 hours made no significant difference to the proportion of radioactivity bound to protein.

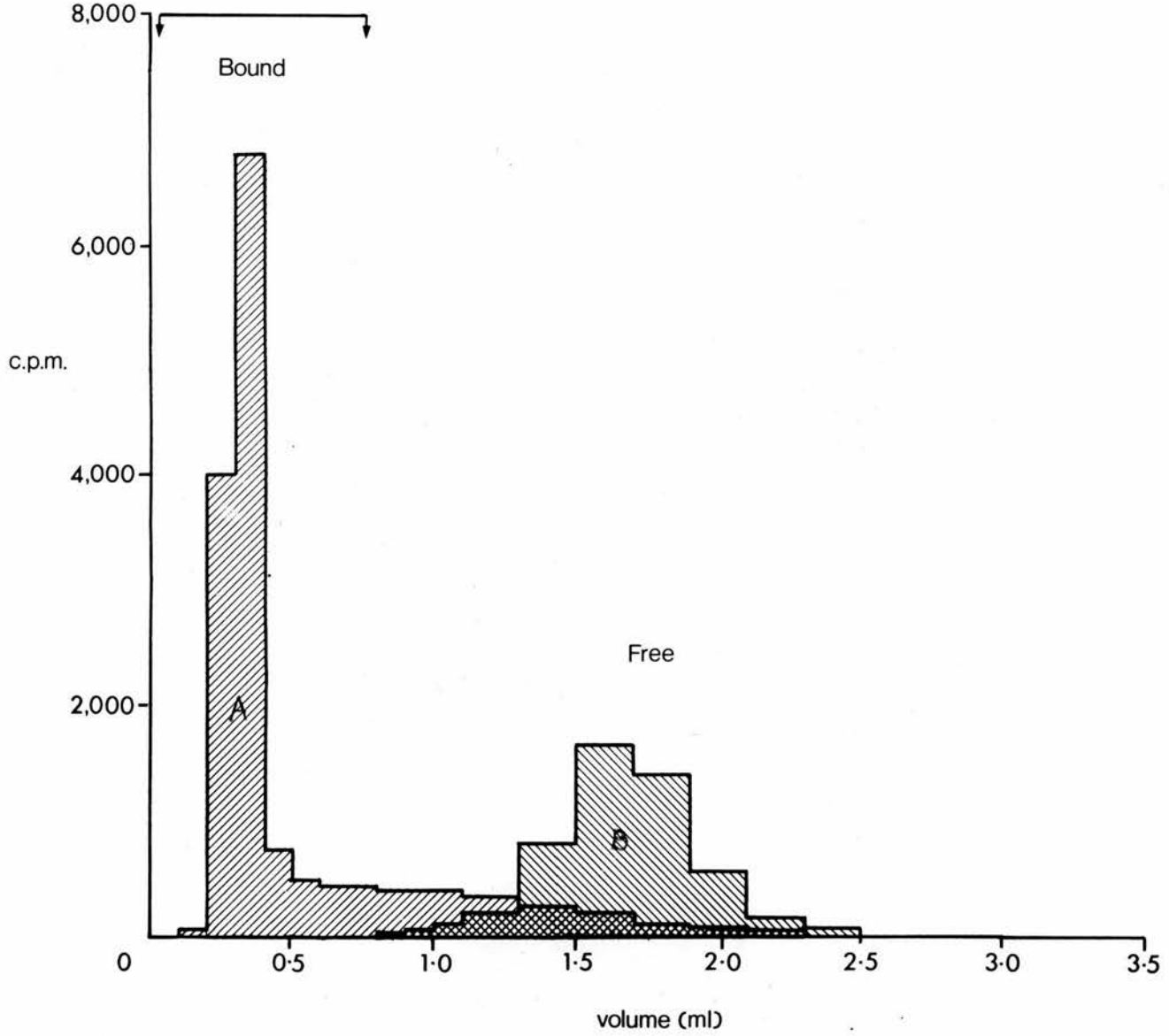
Gel filtration chromatography - Protein bound steroid was separated from steroid free in solution by the use of gel filtration. chromatography on 'Sephadex' ( G25 fine, Pharmacia, Uppsala, Sweden ) columns maintained at 0°C. Microcolumns of 'Pyrex' glass tubing, 0.4 cm i.d., 10 cm high, were surmounted by a 3 ml capacity reservoir which could be sealed by a stopper fitting into a ground glass socket. 8 cm columns of defined 'Sephadex' beads were supported by bolting cloth ( No.25T1, Henry Simon, Stockport, Lancashire ) secured over the bottom of the glass tubes by means of a ring of rubber tubing. The dry weight of 'Sephadex' in these columns was about 200 mg.

Sixty such columns were mounted in a 'Perspex' water jacket made by Mr. D. Findlayson ( Department of Botany, Edinburgh University ). Water inlets and outlets were provided and coolant ( 20 l water, 500 ml glycerol ) was circulated by a centrifugal pump ( SP2, Grant Instruments, Barrington, Cambridgeshire ) from another water bath which contained a cooling coil ( Dipcooler, Techne, Duxford, Cambridgeshire ). The temperature of the coolant was maintained at 0°C by a thermostat and variation was less than $\frac{1}{2}$°C.

'Sephadex' columns could be used repeatedly for periods of up to 6 months. After a 'run', they were washed with 10 ml phosphate buffer. Between 'runs' the top of the reservoirs were stoppered, and the bottom of the column was immersed in a tray of water. After much buffer had been run through the columns, they tended to have low and variable flow rates. This problem could be overcome by agitating the columns with a stiff wire, and then allowing the beads to resettle.

A most useful property of these columns is that they do not admit air into the gel bed immediately after the buffer drains to the top of the 'Sephadex'. The bed is disrupted only after columns have been left unattended for an hour or longer. It was, therefore, possible to collect fractions by adding known volumes of buffer to the column reservoirs. Many such columns can be operated by a single technician. The constancy of the size of the collected fraction was tested after 0.5 ml buffer had been added to the top of the columns: the eluates were collected into weighed phials and the mean weight of the collected fractions was 496 $\pm$ 12 ( S.D.) mg, n = 20.

Separation of protein bound from free steroid - The 0.1 ml incubate from assay tubes was transferred to the top of columns by means of a Pasteur pipette. A 0.15 ml aliquot of buffer was used to rinse the assay tube, and these washings were added to the sample on the column. 95.1 $\pm$ 0.9 ( S.D.) % of the radioactivity in an assay tube was transferred to a column by this means ( n = 10 ). The separation of protein bound from free steroid is illustrated in


8,000
6,000
4,000
2,000
0
c.p.m.
Bound
A
Free
B
0·5
1·0
1·5
2·0
2·5
3·0
3·5
volume (ml)

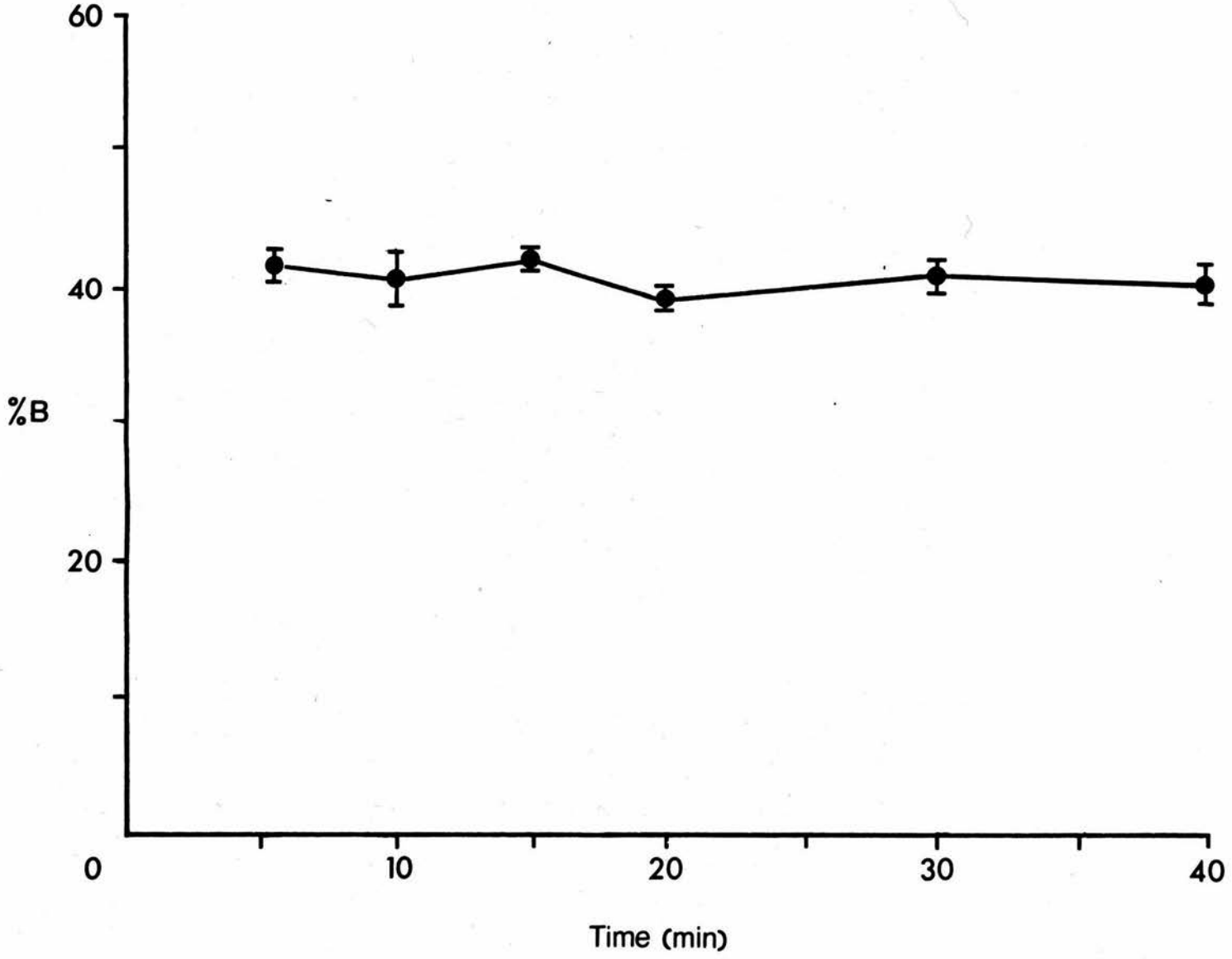
Legend to figure 1

Separation of protein bound from free tritiated progesterone by gel filtration

Sample A; tritiated progesterone was incubated with 0.1 ml of a 1:20 dilution of pregnancy plasma.

Sample B; tritiated progesterone was incubated with 0.1 ml phosphate buffer.

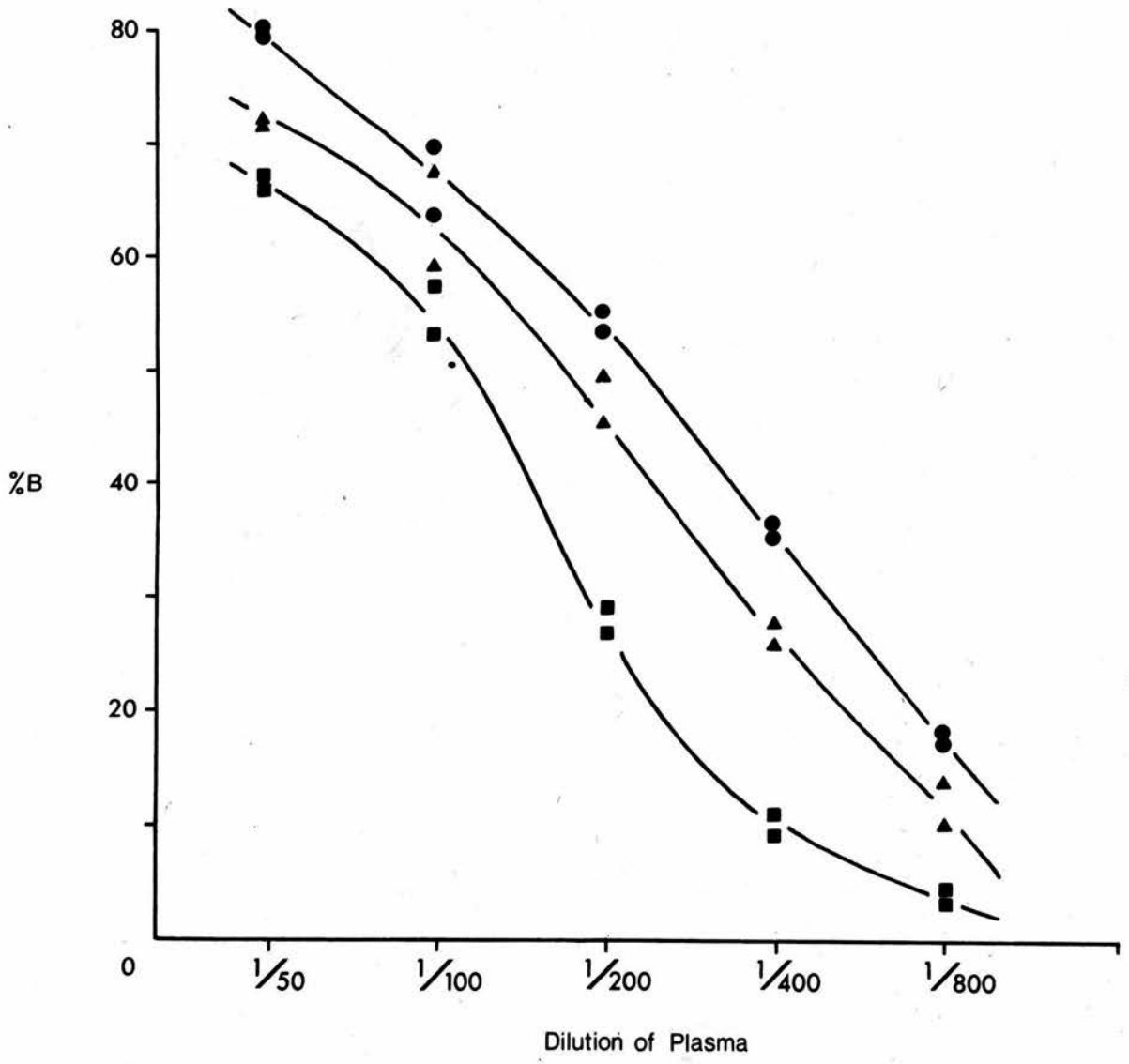
Gel filtration was carried out at 0 °C on a 'Sephadex' ( G25, fine ) column ( 8 cm height, 0.4 cm i.d. ). The eluate was collected in small fractions and the radioactivity in each was determined.


60
40
20
0
%B
10
20
30
40
Time (min)

Legend to figure 2

Failure of 'Sephadex' to strip tritiated progesterone from pregnancy plasma proteins

The 0.1 ml samples of a 1:20 dilution of pregnancy plasma containing 4 ng progesterone and tritiated progesterone were placed on 'Sephadex' columns maintained at 0°C. After various times of incubation in the 'Sephadex' bed, the fraction containing tritiated progesterone bound to protein was eluted. The radioactivity in this fraction was determined and expressed as a percentage of the total radioactivity of the incubate ( % B ). Bars indicate standard errors of the mean of five determinations.


80
60
40
20
0
%B
1/50
1/100
1/200
1/400
1/800
Dilution of Plasma

Legend to figure 3

Variation in steroid binding by pregnancy plasma proteins with temperature and at various dilutions

● columns maintained at 0°C

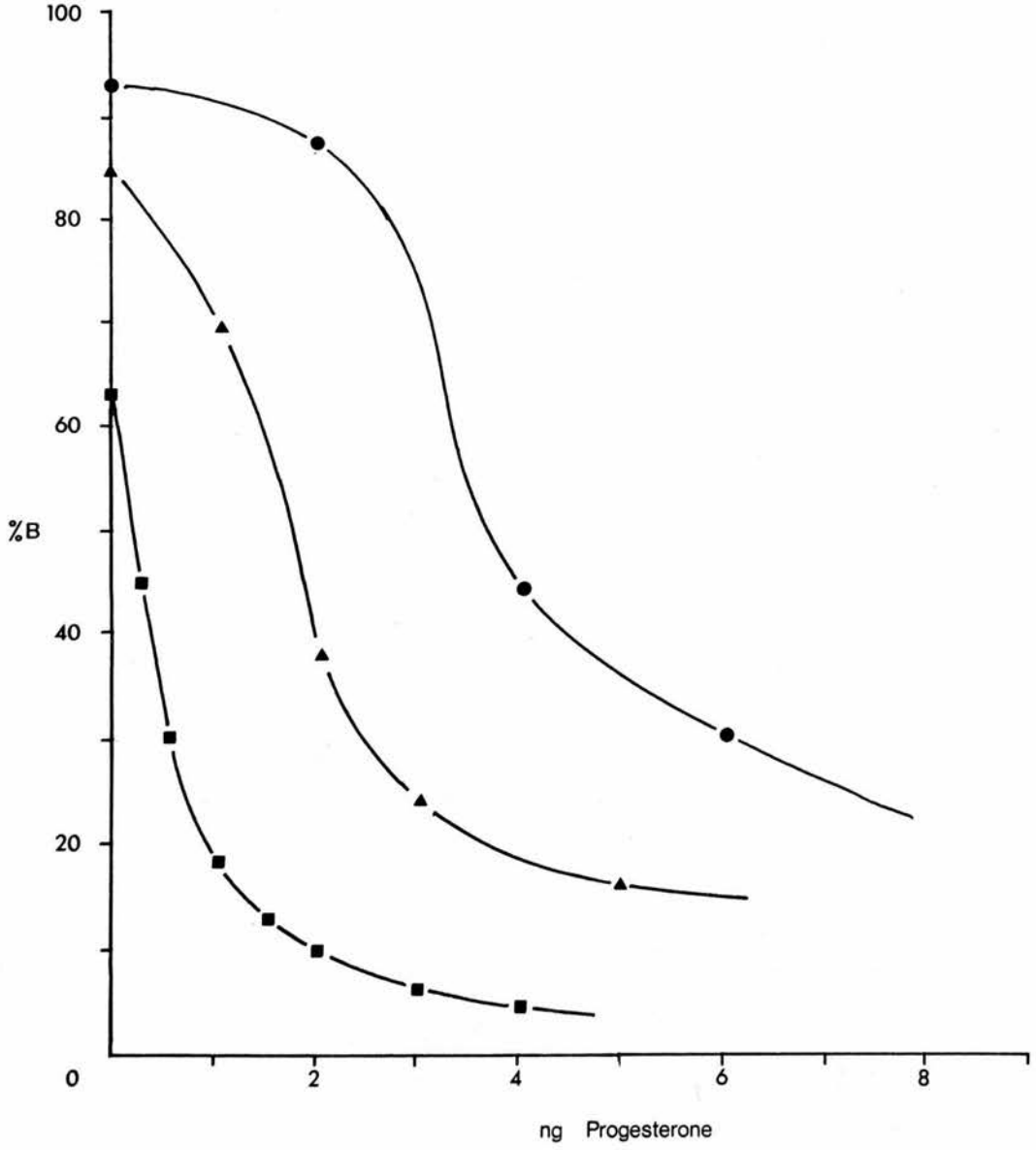
▲ columns maintained at 4°C

■ columns maintained at 10°C

figure 1. The first 0.75 ml of eluate was taken for the 'bound' fraction. When a solution of blue dextran ( Pharmacia ) was chromatographed under the same conditions, the colour was eluted in that fraction. Yoshimi and Lipsett ( 1968 ) reported that 'Sephadex' competes for steroid binding with the plasma proteins. When samples were left in columns for varying times, no evidence was found for this assertion. The proportion of radioactivity eluted in the 'bound' fraction remained constant ( see figure 2 ).

However, temperature had a marked effect on the ability of pregnancy plasma to bind tritiated steroid. So too did the concentration of binding protein. The effect of these two variables is illustrated in figure 3. At room temperature, a 1:20 dilution of pregnancy plasma binds 60 % of tritiated progesterone whereas 1:50 and 1:100 dilutions bind as little as 10 % and 1 % of the total radioactivity in an incubation.

Format of assay 'runs' - 'Standard' amounts of progesterone were derived from a stock solution containing 50 mg/ml in absolute ethanol. A working solution containing 1 µg/ml in ethanol was prepared every month. These solutions were stored at 4°C. On each day of a 'run' of ovarian vein samples, a further dilution containing 100 ng/ml in ethanol was prepared and 20, 30, 40, 60, 80 and 100 µl volumes were dispensed in triplicate into assay tubes to give incubations containing 2, 3, 4, 6, 8 and 10 ng progesterone. When peripheral plasma samples were assayed, a fresh working solution containing 10 ng/ml progesterone in ethanol was prepared. Incubations containing 0.25, 0.5, 1, 1.5, 2, 4,and 6 ng progesterone were prepared, again in triplicate. Tubes containing no added non-radioactive


100
80
60
40
20
0
%B
2
4
6
8
ng Progesterone

Legend to figure 4

Effect of diluting pregnancy plasma on the slope and range of standard curves

In each case the columns were maintained at 0°C.

● 1:20 dilution of plasma

▲ 1:50 dilution of plasma

■ 1:100 dilution of plasma

progesterone were also 'run'. Between 40 and 60 tubes were incubated in each 'run'. The samples were chromatographed on 'Sephadex' micro-columns and the first 0.75 ml of eluate from each column was collected directly into a scintillation phial. The radioactivity in each phial was counted, and the results were expressed as percentages of the total radioactivity in each incubation.

Calculation - From the results of those tubes containing known amounts of progesterone, a standard curve was plotted. The curve was fitted by eye. Amounts of progesterone in unknown samples were read from a standard curve 'run' on the same day. The shape of curves using different dilutions of pregnancy plasma is shown in figure 4.

Determinations of progesterone in ovarian vein plasma were carried out in duplicate using aliquots from the same extract. If the individual values of such duplicates were more than 10 % from the mean estimated concentration of progesterone, the results were rejected, and the aliquots were reassayed. No attempt was made to measure amounts of progesterone above 8 or below 2 ng per incubation. Outside of this region, the standard curve was too shallow to give precise readings when 1:20 dilutions of pregnancy plasma were used as binding protein. If values were outside these limits, the extract was reassayed using an appropriate volume of extract to give a result which could be read off the steep part of the standard curve. The total amount of progesterone in 1 ml ovarian vein plasma was calculated from the size of the aliquot used for the assay. Where tritiated progesterone had been added to plasma samples, the radio-

Table 1.

Stages in the estimation of progesterone concentration of sheep ovarian vein plasma

1. Extract 1 ml with 4×2 ml petrol ether. Wash the extract with 1 ml water. Blow off solvent.
2. Dissolve the extract in 2 ml ethanol.
3. Place a suitable volume of extract in duplicate into assay tubes. Evaporate the solvent in a vacuum oven.
4. Prepare assay tubes containing known amounts of progesterone in triplicate.
5. Incubate 40 - 60 tubes with 0.1 ml 1:20 pregnancy plasma containing 10,000 c.p.m. $^{3}$H-progesterone at 37°C for 15 min and then at 0°C for 2h.
6. Separate protein bound from free by 'Sephadex' gel filtration.
7. Count the radioactivity in each of the first 0.5 ml eluates.
8. Calculate the fraction of $^{3}$H-progesterone bound to protein in each tube.
9. From the results of tubes containing known amounts of progesterone, plot a standard curve.
10. Read off the mass of progesterone in unknown tubes from the standard curve.
11. Calculate the total progesterone content of 1 ml plasma and express the result in $\mu g$ / ml.

Table 2.

Stages in the estimation of progesterone concentration of sheep peripheral plasma

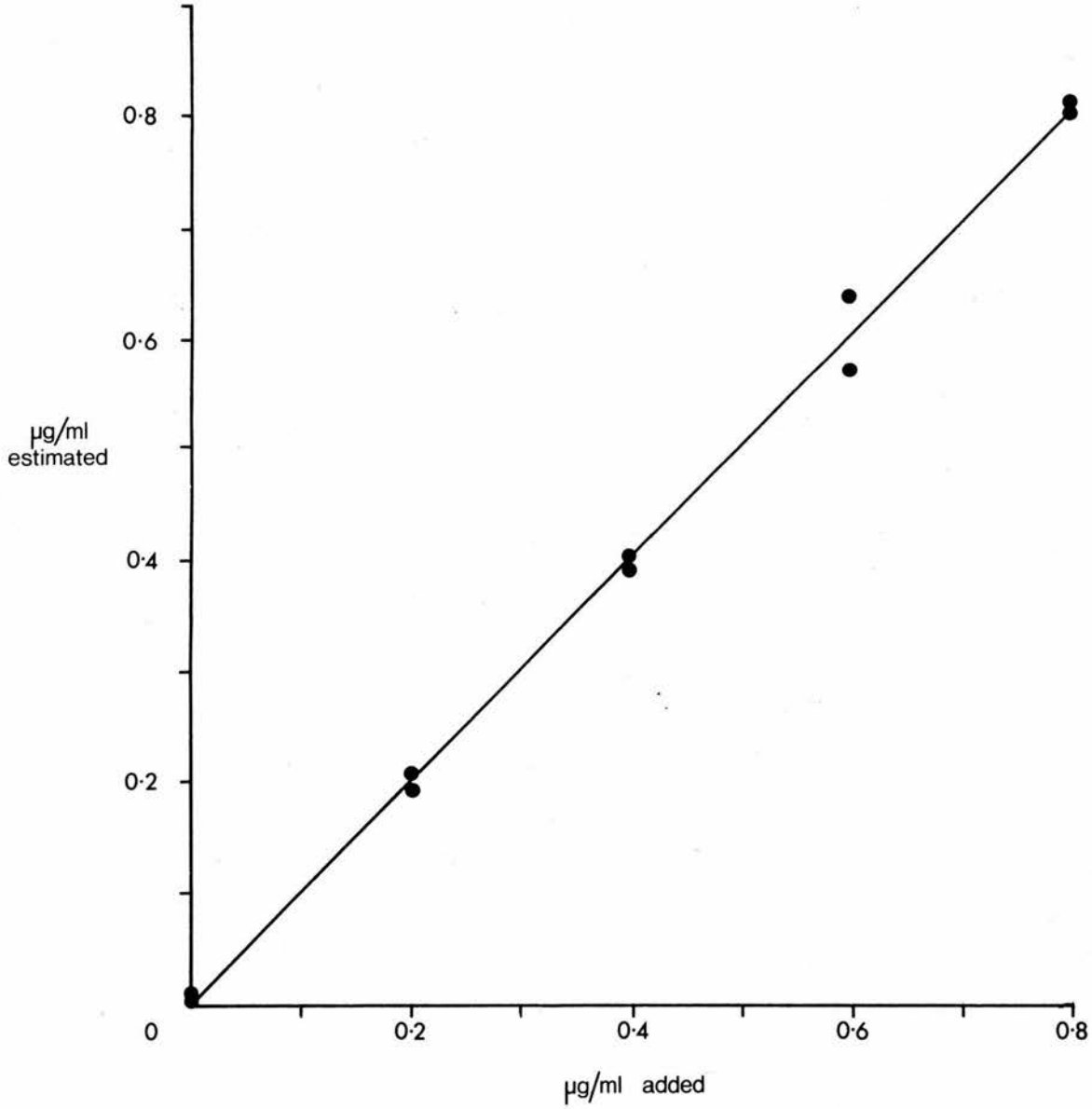
1. Extract 1 ml with 2×4 ml petrol ether. Wash the extract with 1 ml water.
2. Transfer the extract to an assay tube. Blow off solvent.
3. Prepare assay tubes containing known amounts of progesterone in triplicate.
4. Incubate 40 - 60 tubes with 0.1 ml 1:50 pregnancy plasma containing 5,000 c.p.m. $^{3}$H-progesterone and 0.1 % gelatin at 37°C for 15 min and then at 0°C for 2h.
5. Separate protein bound from free by 'Sephadex' gel filtration.
6. Count the radioactivity in each of the first 0.5 ml eluates.
7. Calculate the fraction of $^{3}$H-progesterone bound to protein in each tube.
8. From the results of tubes containing known amounts of progesterone, plot a standard curve.
9. Read off the mass of progesterone in unknown samples by use of the standard curve.

activity in a one tenth aliquot was determined and the estimate was corrected for the procedural losses. Peripheral plasma samples were assayed once. No correction was made for procedural losses, and the concentration of progesterone was read directly off the standard curve because the whole extract of 1 ml plasma was used for the assay. An Olivetti 'Programma 101' desk top computer was used in the calculation phase of the sssay. Small programmes were written as required.

Steps in the progesterone assay are outlined in tables 1 and 2.

Results

An assay blank is here defined as a detectable, significant, response of the test system to non-specific substances in plasma or in the reagents used for preparing the extracts. Using a 1:20 dilution of pregnancy plasma for the assay, no detectable blank was found from the residue left after evaporation of 20 ml petrol ether, 10 ml absolute ethanol or 10 ml diethyl ether. The diethyl ether eluate from a 4 $cm^2$ spot of silica gel off a TLC plate gave a high and erratic blank unless alumina chromatography had been performed on the extract. A peripheral plasma sample was collected from ewes on the same day as infusion experiments. These samples were assayed using the same size of aliquot of the extract as was used for the ovarian vein plasma samples. In no case was progesterone detected in these aliquots using a 1:20 dilution of pregnancy plasma.

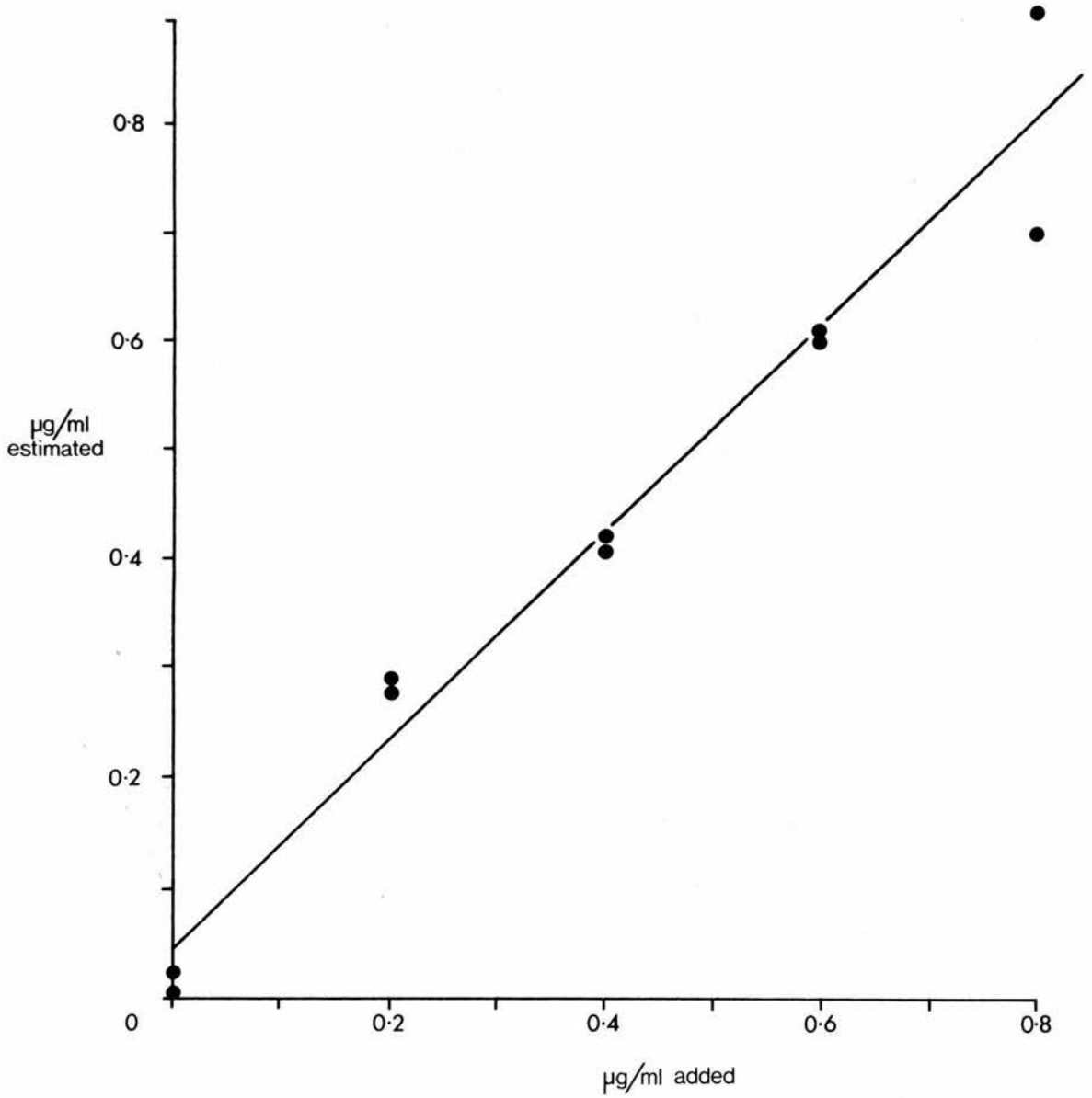

0·8
0·6
µg/ml
estimated
0·4
0·2
0
0·2
0·4
0·6
0·8
µg/ml added

Legend to figure 5

Accuracy of CPB method for ovarian vein plasma samples

Various amounts of progesterone were added to peripheral plasma taken from a castrate ewe. The samples were analysed according to the instructions in table 1. The estimates were individually corrected for procedural losses from the recovery of radioactivity after the addition of 1,000 c.p.m. tritiated progesterone to each sample.

slope = 1.01; intercept = 0.00; r = 0.998; n = 10.

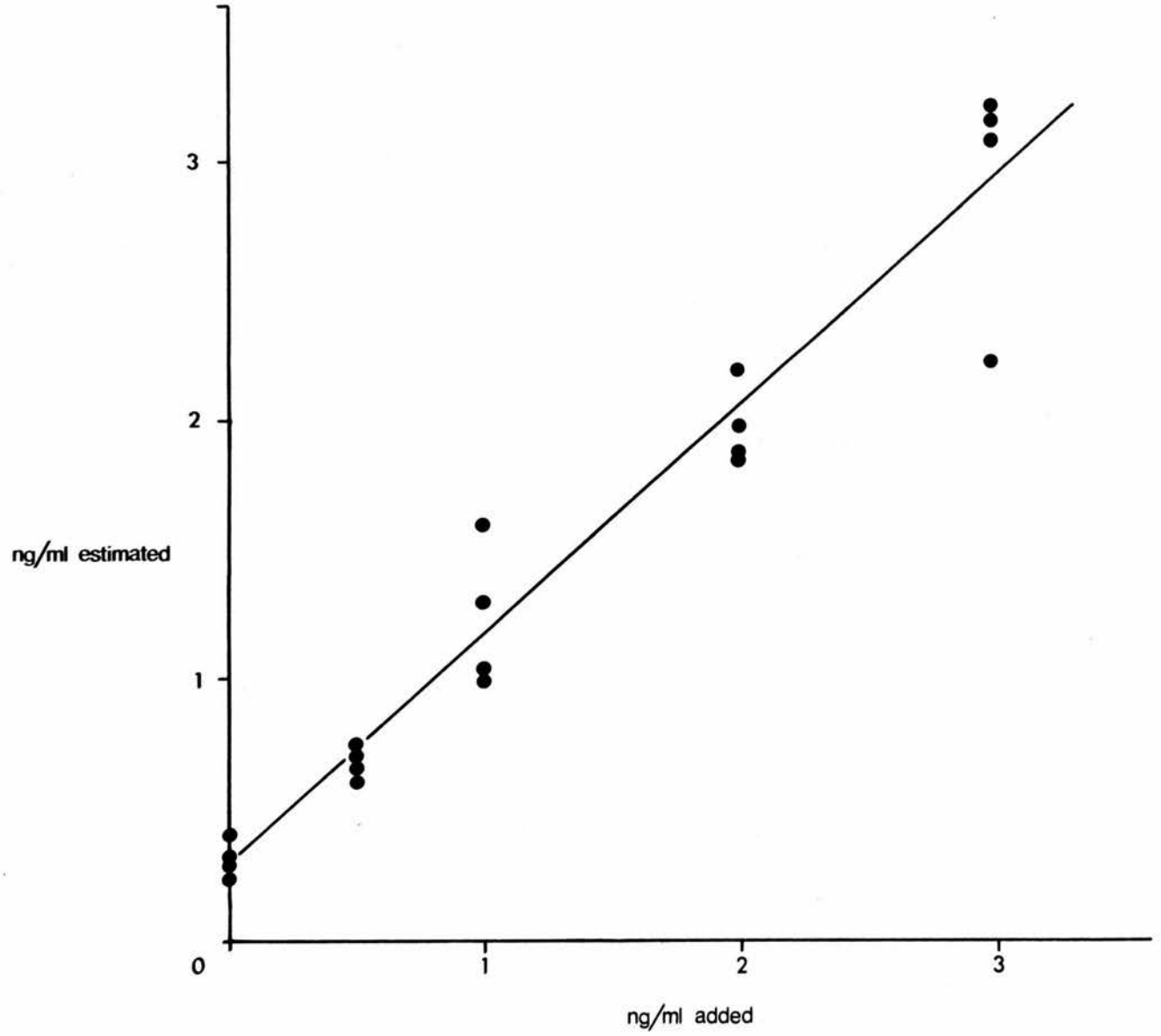

0·8
0·6
µg/ml
estimated
0·4
0·2
0
0·2
0·4
0·6
0·8
µg/ml added

Legend to figure 6

Accuracy of CPB method when extracts have been purified by TLC and alumina column chromatography

Various amounts of progesterone were added to peripheral plasma taken from a castrate ewe. The 1 ml samples were purified and then analysed for progesterone by CPB. The estimates were individually corrected for procedural losses from the recovery of radioactivity after the addition of 1,000 c.p.m. tritiated progesterone to each sample.

slope = 0.948; intercept = 0.05; r = 0.981; n = 10.


3
2
1
0
ng/ml estimated
1
2
3
ng/ml added

Legend to figure 7

Accuracy of CPB method for sheep peripheral plasma samples

Various amounts of progesterone were added to peripheral plasma taken from a castrate ewe. The samples were assayed according to the instructions in table 2. The estimates were not corrected for procedural losses.

slope = 0.86; intercept = 0.30; r = 0.97; n = 20.

When a 1:50 dilution of pregnancy plasma was used for the assay of extracts of 1 ml peripheral plasma, a number of substances gave detectable responses. Although 10 ml diethyl ether from freshly opened bottles was clean, it gave a blank if it had been exposed to the atmosphere for some time. No blanks were given by 2 ml ethanol or 10 ml petrol ether. 'Negative' blanks - values of per cent bound lying above the results of tubes containing no added progesterone - were encountered if the extraction step had been performed carelessly and protein from the plasma had found its way into assay tubes. Presumably it too bound tritiated progesterone.

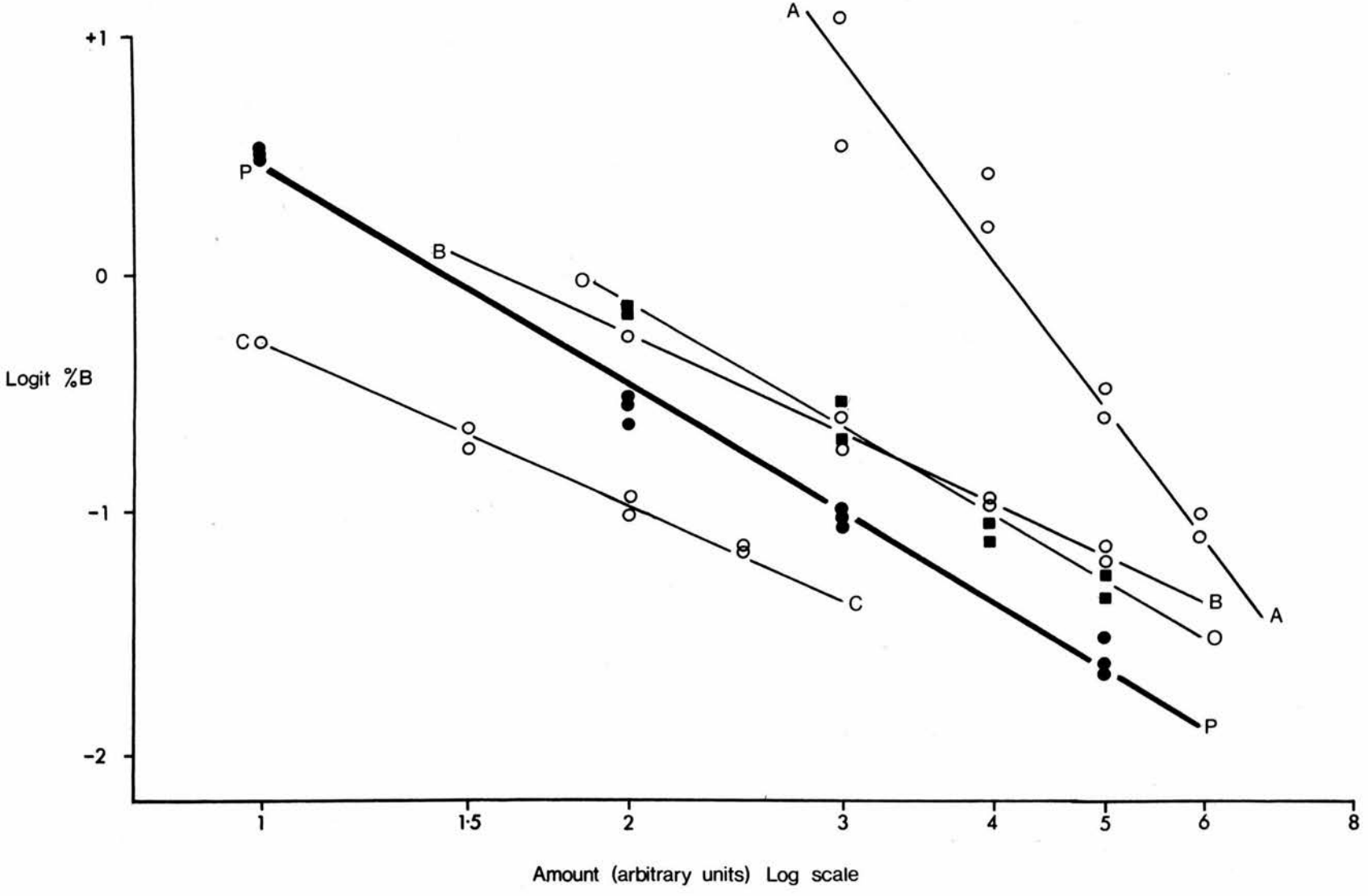
On each day that peripheral plasma samples were measured, 1 ml of a pool of plasma taken from a castrate ewe and 1 ml distilled water were also extracted and assayed. The estimates were: for distilled water, 0.38 $\pm$ 0.158 ( S.D.) ng ( n = 10 ); and for the plasma, 0.53 $\pm$ 0.136 ( S.D.) ng ( n = 16 ). Extraction of increasing amounts of distilled water gave a displacement curve much more shallow than that of progesterone, whereas the displacement curve for increasing amounts of castrate ewe's plasma was much the same as that for progesterone.

The accuracy of the method was assessed by measuring pools of plasma from a castrate ewe to which known amounts of progesterone had been added. The results for assays conducted according to table 1 and then corrected for procedural losses are shown in figure 5. When a TLC step was added, the results were only slightly more variable ( see figure 6 ). When peripheral plasma samples were assayed according to the method in table 2, the results were as shown in figure 7.

Within batch precision was tested by repeatedly assaying pools of ovarian vein plasma. Ten 1 ml samples of each pool were assayed according to table 1 and the results were corrected for the procedural losses estimated from the recovery of added tritiated progesterone. The results were: pool A, 0.35 ± 0.023 ( S.D.) µg/ml; pool B, 0.48 ± 0.020 ( S.D.) µg/ml; and for pool C, 0.83 ± 0.027 ( S.D.) µg/ml. Without correction for losses the results were: pool A, 0.32 ± 0.016 ( S.D.) µg/ml; pool B, 0.39 ± 0.021 ( S.D.) µg/ml; and pool C, 0.76 ± 0.026 ( S.D.) µg/ml. The coefficients of variation for the uncorrected estimates are not significantly higher than those for the corrected ones.

Between batch precision was tested by means of 'quality control' samples that were assayed every time that a 'run' was done. A pool of ovarian vein plasma, D, was assayed according to table 1 on 22 different occasions. The result was 0.24 ± 0.032 ( S.D.) µg/ml. Two pools of peripheral plasma were assayed on 16 occasions according to table 2. Pool E ( castrate ewe's plasma with 0.2 ng/ml added progesterone ) gave 0.74 ± 0.132 ( S.D.) ng/ml, and pool F, ( castrate ewe's plasma with 1 ng/ml added progesterone ) gave 1.28 ± 0.293 ( S.D.) ng/ml.

Three steroids were added in concentrations of 1 µg/ml to plasma taken from a castrate ewe. These samples were assayed as outlined in table 1, and the following results were obtained: the sample containing 17∝hydroxyprogesterone, 0.55 µg/ml; the sample with 20∝dihydroprogesterone, 0.15 µg/ml; and the sample with pregnenolone, 0.15 µg/ml. No progesterone was detected when a solution


+1
0
-1
-2
Logit %B
A
B
C
P
1
1·5
2
3
4
5
6
8
Amount (arbitrary units) Log scale

Legend to figure 8

Logit-log displacement curves for progesterone, extracts of ovarian vein plasma and three steroids

● progesterone standards

■ extract of ovarian vein plasma

o other steroids

A 17∝-hydroxyprogesterone

B 20∝-dihydroprogesterone

C pregnenolone

P progesterone

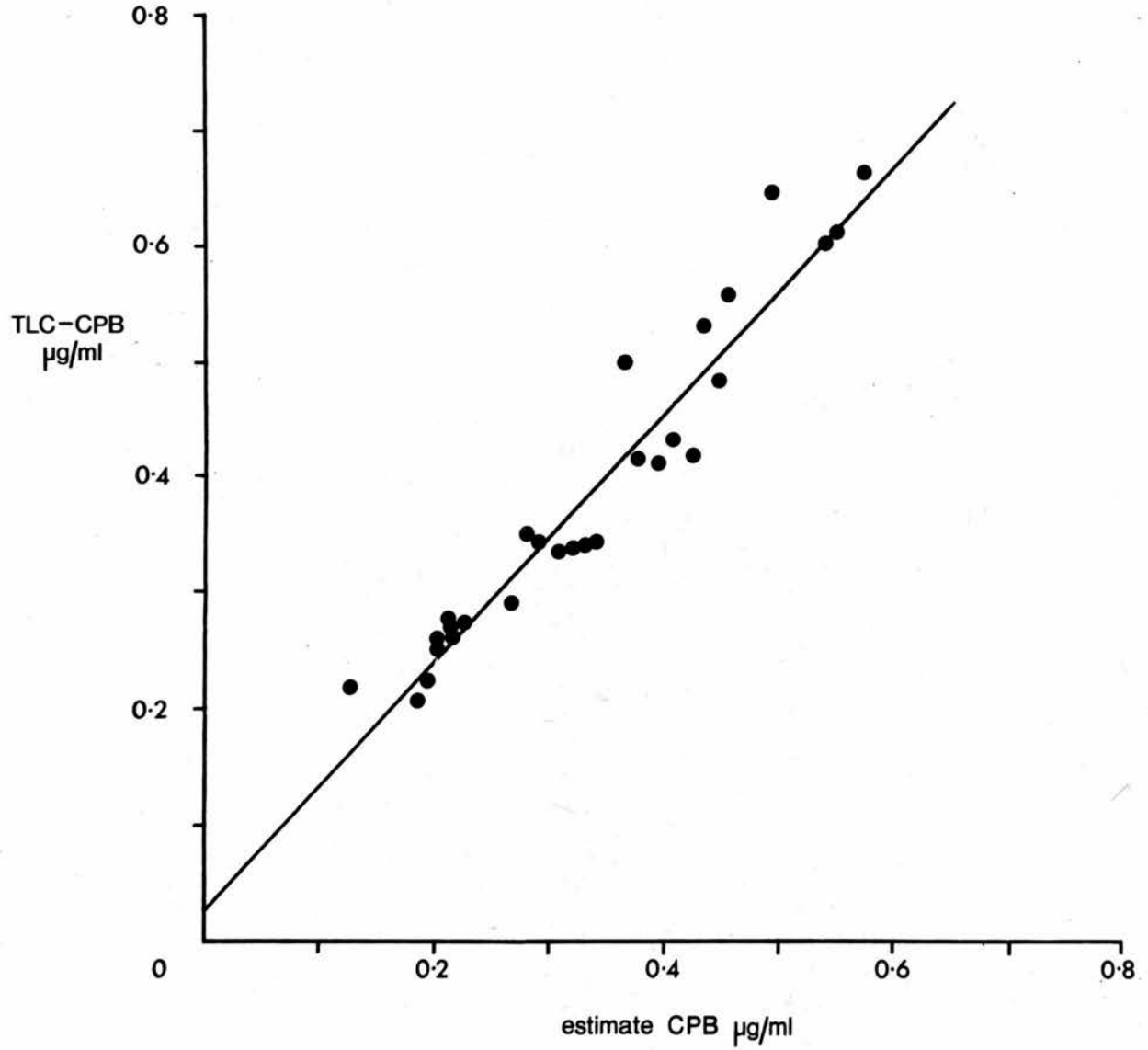
O extract of ovarian vein plasma

of heparin was assayed.

Aliquots of various amounts of an extract of ovarian vein plasma were assayed. The same was done on extracts of plasma from a castrate ewe to which 17∝-hydroxyprogesterone, 20∝dihydroprogesterone, and pregnenolone had been added. The displacement curves of each extract were plotted on a logit-logarithmic scale ( Rodbard, Bridson & Rayford, 1969 ), and compared: see figure 8 ). The slope of the curve for progesterone is parallel to that of the ovarian vein extract but much less steep than that from the extract of plasma containing 17∝-hydroxyprogesterone.

A 5 ml sample of ovarian vein plasma was extracted with petrol ether and subjected to TLC. Of the various spots analysed, 3.4 µg was found in the spot moving with the same Rf as progesterone and the displacing activity of all other spots was equivalent to a mere 0.07 µg progesterone. Of that activity, 0.013 µg had the same Rf as 20∝-dihydroprogesterone. Nine tenths of the displacing activity in ovine peripheral plasma had the same Rf as progesterone on TLC. An extract of a pool of peripheral plasma taken from female rats showed two spots of displacing activity; one had the same Rf as progesterone, the other, the same Rf as 20∝-dihydroprogesterone on TLC.

Petrol ether extracts of ovarian vein plasma samples taken during experiment 3 were assayed both before and after TLC. Both estimates were corrected for procedural losses. The correlation between the two estimates was high ( see figure 9 ).

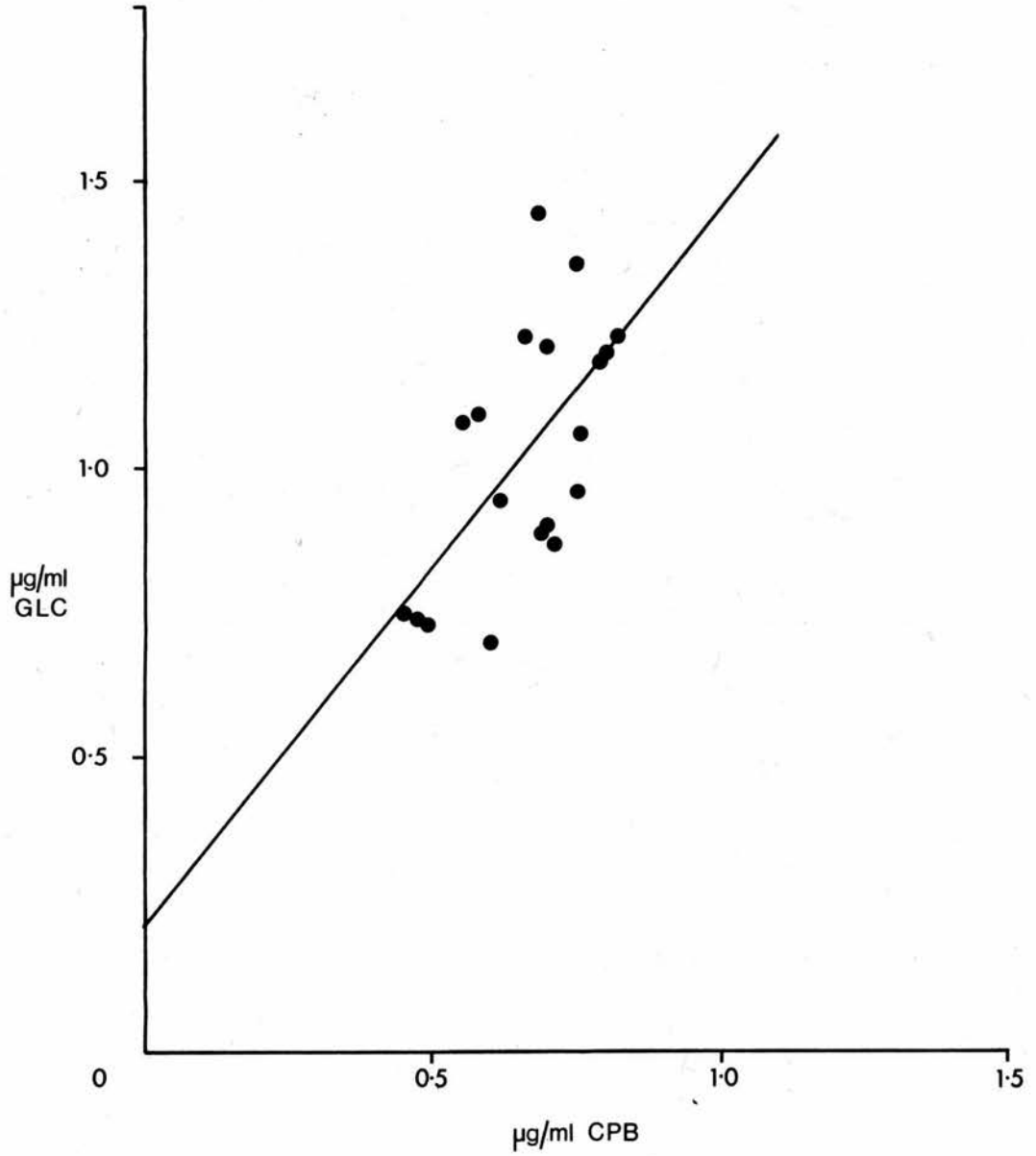

0·8
0·6
0·4
0·2
0
TLC–CPB
µg/ml
0·2
0·4
0·6
0·8
estimate CPB µg/ml

Legend to figure 9

Correlation between estimates obtained by CPB before and after purification of extracts

Extracts of ovarian vein samples were assayed by CPB before and after chromatography on thin layer silica gel and an alumina column. Both estimates were corrected for procedural losses.

slope = 1.07; intercept = 0.03; r = 0.96; n = 28.


1·5
1·0
0·5
0
µg/ml
GLC
0·5
1·0
1·5
µg/ml CPB

Legend to figure 10

Correlation between estimates obtained by CPB and GLC methods

GLC and quantitation were carried out as described in the text. CPB assay was performed according to the instructions in table 1. All estimates were corrected for procedural losses. All samples were of sheep ovarian vein plasma.

slope = 1.23 $\pm$ 0.37 (S.E.); intercept = 0.22 $\pm$ 0.18 (S.E.); r = 0.622; n = 19.

Table 3.

Comparison of estimates of progesterone concentration in nine sheep peripheral plasma samples by four radiodisplacement assays

| Nature of sample | Results ng / ml | | | |
|---|---|---|---|---|
| | Assay 1 | Assay 2 | Assay 3 | Assay 4 |
| Luteal phase | 1.3 | - | - | 1.8 |
| Castrate | 0.55 | 0.63 | 0.94 | 0.7 |
| Castrate + 5 ng/ml | 2.9 | 4.9 | 4.0 | 4.6 |
| Luteal phase | 1.7 | 2.1 | 2.1 | 2.1 |
| Castrate + 0.5 ng/ml | 2.4, 0.5 | 0.6 | 0.97 | 0.8 |
| Luteal phase | 1.3 | 1.3 | 1.9 | 1.9 |
| Castrate + 1 ng/ml | 1.1 | 1.3 | 1.1 | 1.1 |
| Castrate + 0.2 ng/ml | 0.63 | 0.8 | 1.0 | 0.8 |
| Luteal phase | 2.1 | 2.6 | 2.3 | 2.6 |

Assay 1 was conducted by Mr. K. McNatty ( M.R.C. Reproductive Biology Unit, Edinburgh). He used a RIA method modified after Thorneycroft and Stone (1972) and an antibody from Mr. B. Dighe.

Assay 2 was conducted by Christine Bishop ( Beechams Medicinal Research Centre, Harlow, Essex. She used a RIA method employing an antibody from Dr. B.V. Caldwell ( Bishop & Flack, unpublished ).

Assay 3 was conducted in the department of Obstetrics & Gynaecology, University of Glasgow ( Dodson, Coutts & MacNaughton, 1973 ).

Assay 4 was the CPB method outlined in table 2.

Nineteen ovarian vein plasma samples from experiment 7 were assayed by both GLC and CPB. The correlation between estimates of the progesterone concentrations obtained by these two methods is shown in figure 10.

Nine samples of peripheral ovine plasma were circulated to three other workers who have developed CPB or RIA methods for progesterone. The results are shown in table 3.

The ingredients that go to make up practicability are: reliability, simplicity, speed and cost. Very few 'runs' were entirely lost. However, about 3 % of individual results were rejected because they were very far from expected values and could not be confirmed. When peripheral samples were 'run', the most frequent gross error was a complete displacement of the added radioactivity. This method is among the most simple of those published for progesterone. By reason of the two factors mentioned above, it was successfully taught to two technicians in the laboratory within one week. About 1,800 ovarian vein samples a year can be measured according to the scheme in table 1 by one technician. This figure takes account of assistance in in washing the glassware and the time taken for holidays and routine laboratory maintenance. About 3,600 peripheral plasma samples can be handled in one year under the same conditions. The cost per assay is very difficult to assess, but the share of capital equipment used solely for this assay cost between £3,500 and £4,000 at 1973 prices. One laboratory size 30 $m^2$ was used. The running costs are estimated to be between £2,000 and £2,500 p.a. for salaries and about £700 p.a. for chemicals, other consumables and service contracts, all at 1973 prices.

## Discussion

The method of sample preparation is of as great importance as the detection system in any sensitive assay for steroids. Even when extracts are purified, non-specific substances are frequently found to interfere with the detection system. In the assay described here, the procedural losses were not estimated individually, and the petrol ether extract was not purified. In this assay, then, the extraction step is of even greater importance than in most other assays.

Petrol ether is a solvent specific for less polar steroids. In particular, cortisol is not extracted and this confirms earlier work ( Murphy, 1967; Johansson, 1969; Thorburn, Bassett & Smith, 1969 ). Some 17$\alpha$-hydroxyprogesterone and 20$\alpha$-dihydroprogesterone is extracted and these steroids are considered later. The recovery of progesterone was very high and uniform. The decision to drop the correction of estimates individually for procedural losses is fully vindicated by the data showing that this omission has no detrimental effect upon precision. The three steps used in preparing extracts are more time consuming than the procedure used by Johansson ( 1969 ). However, the combination of two successive extracts improves the precision of the recovery of steroid. The water wash improves the specificity by removing some 17$\alpha$-hydroxyprogesterone and 20$\alpha$dihydro-progesterone as well as protein accidentally transferred with the organic phase.

In early CPB methods, dialysis was used to separate protein bound from free steroids, but this is too time consuming ( Murphy, Engelberg & Pattee, 1963 ). It is not suitable if many samples are to be analysed.

The use of solid adsorbents, especially inorganic silicates, was pioneered by Murphy ( 1967 ). Numerous published methods for progesterone have used this procedure ( for example: Neill, Johansson, Datta & Knobil, 1967; Strott & Lipsett, 1968; Johansson, 1969; Martin, Cooke & Black, 1970 ), but difficulties have been reported by a Swedish group ( de Souza, Williamson, Moody & Diczfalusy, 1970 ). They found some batches of florisil were unsatisfactory. Dextran coated charcoal has been used extensively for RIA of steroids including progesterone ( Thorneycroft & Stone, 1972; Furr, 1973 ). Unfortunately, the binding affinity of progesterone for pregnancy plasma is low, and charcoal continues to strip steroid from proteins. The solubility characteristics of corticosterone binding globulin make the use of ammonium sulphate precipitation, such as has been used when antibodies have been used as binding protein ( Furuyama & Nugent, 1971 ), unsuitable. Although adsorption methods are faster than gel filtration, they are less reliable. The method chosen is that reported by Yoshimi and Lipsett ( 1968 ). A water jacket was used to cool the 'Sephadex' columns because cold room facilities were not available. This modification has the advantage that the operator may work at room temperature, an important consideration for precise work. The data shown in figure 2 does not support the claim that 'Sephadex' strips steroid from binding protein ( Yoshimi & Lipsett, 1968 ), but temperature was a very important variable ( see figure 3 ).

RIA and CPB dose response curves may be linearized by various transformations ( Rodbard, Bridson & Rayford, 1969 ). The advantages of linearization can be summarized: the best fit graph can be calculated and subjective bias in 'fitting by eye' eliminated; points well clear of the best fit curve may be rejected after the application of sound statistical principles; and finally the confidence limits of estimates can be calculated from the within assay variation. However, transformation of data creates inhomogeneity of variance and further calculations where the data is weighted according to its variance have to be performed. Even when a desk top computer is used, these manipulations take a long time. When the logit-log transformation was applied to standard curves, statistical tests revealed them to be significantly non-linear. For these reasons the dose response curves were not routinely linearized. The variation in estimates was tested directly by means of 'quality control' samples ( Rodbard, 1971 ). Subjective bias was reduced by reading off results of unknown samples before their identity had been noted.

The accuracy of an analytical method may be defined as the ability that it possesses to estimate the 'true' concentration of the substance being measured. The components of accuracy are : specificity, precision and repeatability.

Non-steroidal interfering factors are eluted from chromatographic supports. I found that the material eluted from silica gel was removed by alumina column chromatography confirming the finding of Mayes and Nugent ( 1968 ). Others have noted that supports require extensive washing if they are to be successfully

used in CPB assays ( Neill, Johansson, Datta & Knobil, 1967; Yoshimi & Lipsett, 1968; Martin, Cooke& Black, 1970 ). The 'method interfering factors' eluted from silica gel TLC plates have been investigated ( de Souza, Williamson, Moody & Diczfalusy, 1970 ) and it is clear that there is no ideal way of compensating for their effect. Recently, 'Sephadex-LH20' has been reported to effect the clean separation of steroids without introducing interfering factors ( Murphy, 1971 ). However, it was wished to develop a method free from chromatography steps and the problems of correcting for procedural losses.

The specificity of steroid binding by human corticosteroid binding globulin has been reported ( Murphy, 1969 ). Most steroids with the 4-en-3-one group and 17$\alpha$-hydroxy or 20-keto group bind. It was felt that the steroids of this group which were most likely to appear in sheep plasma were cortisol, corticosterone, 17$\alpha$-hydroxyprogesterone, pregnenolone and 20$\alpha$-dihydroprogesterone. Cortisol is the main adrenal steroid in sheep ( Bush & Ferguson, 1953 ) and the progesterone derivatives are secreted in comparatively low amounts by the sheep ovary ( Short, McDonald & Rowson, 1963; Baird, Goding, Ichikawa & McCracken, 1968 ). Cortisol is not extracted by petrol ether, and the amounts of other interfering progestins are greatly reduced in comparison with progesterone. TLC showed that almost the entire displacing activity of a petrol ether extract of ovarian vein plasma moved with the same Rf as progesterone. Furthermore, the displacement curve of such an extract lay parallel to one produced by progesterone. The material being assayed behaves like proges-

terone in two important respects and it is unlike the other adrenal or ovarian steroids investigated. It is concluded that this assay does in fact measure progesterone in samples of sheep peripheral plasma with negligible interference from other steroids. These findings confirm previous work ( Thorburn, Bassett & Smith, 1969 ).

The precision of this assay is fully comparable with that of others using CPB or RIA techniques. Like other workers, I found that between-batch variation was greater than within-batch variation ( Rodbard, 1971 ). Theoretical models have been proposed for predicting optimal incubation conditions for assay ( Ekins & Newman, 1970; Feldman & Rodbard, 1971; Yalow & Berson, 1971 ). The concentration of binding protein used here for assaying peripheral samples is within the range predicted by Ekins and Rodbard, but the mass of tritiated steroid was far below their recommendations. However, both these workers made the assumption that counting error contributed significantly to the overall variation. In this assay, the specific activity of the tritiated steroid is such that the mass of tritiated steroid being used in an assay is far below that being measured.

Sensitivity - the lower detection limit - of the assay was of no importance when ovarian vein plasma samples were being assayed. In these samples, no effort was made to estimate levels below 10 ng/ml. The limit of sensitivity for measuring progesterone in peripheral plasma samples is about 0.8 ng/ml. This limit could be reduced by measuring samples in duplicate. The estimated concentration of progesterone in plasma obtained from a castrate ewe is 0.5 ng/ml.

Three other laboratories that also measured this pool found a blank ( see table 3 ), and one of these assays ( number 1 ) is said to employ a highly specific antiserum. However, the expected concentration of progesterone in peripheral plasma may be calculated from the metabolic clearance rate and adrenal secretion rate of this hormone. Since this calculated value is only 0.04 ng/ml, it may be inferred that this displacing activity in castrate ewe's plasma is not identical to progesterone, or that this steroid is produced by conversion from other steroids in the blood.

When known amounts of progesterone were added to plasma obtained from a castrate ewe and the samples assayed, the estimates were in close agreement with the amounts of added steroid. These experiments strongly confirm the accuracy of the assay. Moreover, the estimates were not significantly different from those found using a GLC method. Other workers using progesterone assays were able to confirm my estimates on a small number of samples.

It is concluded that the method is specific, accurate, and sufficiently rapid for the purposes of this project. The precision compares favourably with that reported by others. The sensitivity is less than that reported by workers using RIA for progesterone ( Abraham, Swerdloff, Tulchinsky & Odell, 1971, Furuyama & Nugent, 1971; Furr, 1973 ), but this assay is capable of distinguishing ewes with functional corpora lutea from those without on the basis of of their peripheral progesterone concentrations.

Table 4.

Details of ewes prepared with transplanted ovaries

| Ewe number | Breed | Year of birth | Number of lambings | Dates of surgery for neck loop | Dates of surgery for transplant |
|---|---|---|---|---|---|
| CB69 | Merino-Blackface | 1964 | 3 | 9-12-68 | 7-2-69 |
| CB94 | Merino-Blackface | 1965 | 1 | 28-11-68 | 5-2-69 |
| D304 | Merino | 1966 | 1 | 26-5-69 | 19-9-69 |
| D305 | Merino | 1966 | 2 | 10-10-69 | 19-12-69 |
| D314 | Merino | 1966 | 3 | 16-10-69 | 24-4-71 |
| D342 | Merino | 1967 | 1 | 9-8-69 | 12-11-69 |
| MX9 | Merino-Cheviot | 1968 | 1 | 17-10-69 | 11-2-71 |

## THE EXPERIMENTAL EWES

### Surgery

Surgical methods for transplanting the sheep ovary have been described in detail ( Goding, McCracken & Baird, 1967 ), and the next few pages merely summarize these procedures.

Healthy Merino and Merino-cross ewes were selected for this study after they had a proven reproductive performance. The ages, the breeds, the obstetric histories, and the dates of surgery are indicated in table 4.

On the day before surgery, ewes were brought indoors and starved overnight. The surgery for ovary transplantation was carried out in two stages. At both, anaesthesia was induced with thiopentone and maintained by closed circuit halothane. At the first stage, the right jugular vein and right carotid artery was exposed and cleared of all attachments and tributaries over a length of 25 cm. A skin tube was fashioned to enclose the vessels and a central skin bridge with a pouch was made to receive the transplant.

Two or three months later, the ovary was transplanted. The neck loop was reopened, and the two blood vessels in it were exposed. Simultaneously, the abdomen was opened and the blood vessels to the left ovary were identified and cleared. After the ewe had been heparinized, the middle uterine vein was ligated at a position just before it received the left ovarian vein. The aorta was closed

around the origin of the ovarian artery, and a patch of the aorta containing the orifice of the ovarian artery was excised. The aorta was closed by lateral suture, the right ovary removed, and its vessels ligated. The abdominal wound was closed. A small hole was made in the right carotid artery, and the patch of aorta containing the origin of the ovarian artery was used to close this hole. When this anastomosis was complete, bleeding occurred from the uterine vein. This vessel was joined to the jugular vein by end-to-side anastomosis. When bleeding had stopped, the wound was closed. The transplant operation took about 3 hours. The ovary was without a blood supply for about 10 minutes.

Care of the ewes

Ewes were cared for by the staff at Dryden Field Station, Animal Breeding Research Organization, Roslin, Midlothian under the supervision of Dr. R.B. Land.

After operations, the ewes were kept indoors. Fluid from the neck wound was collected into an evacuated drain. This was removed as soon as the reaction had subsided. During the summer months, ewes were allowed out to pasture. In the winter, they were kept in loose housing. While they were indoors, artificial light was provided during daylight hours. The ewes were fed hay when they were not in pasture. Concentrates were added to their feed when it was poor. On the day before infusions, the ewe was transferred to a smaller pen with a companion. The presence of the latter is important because isolated sheep become restless and agitated.

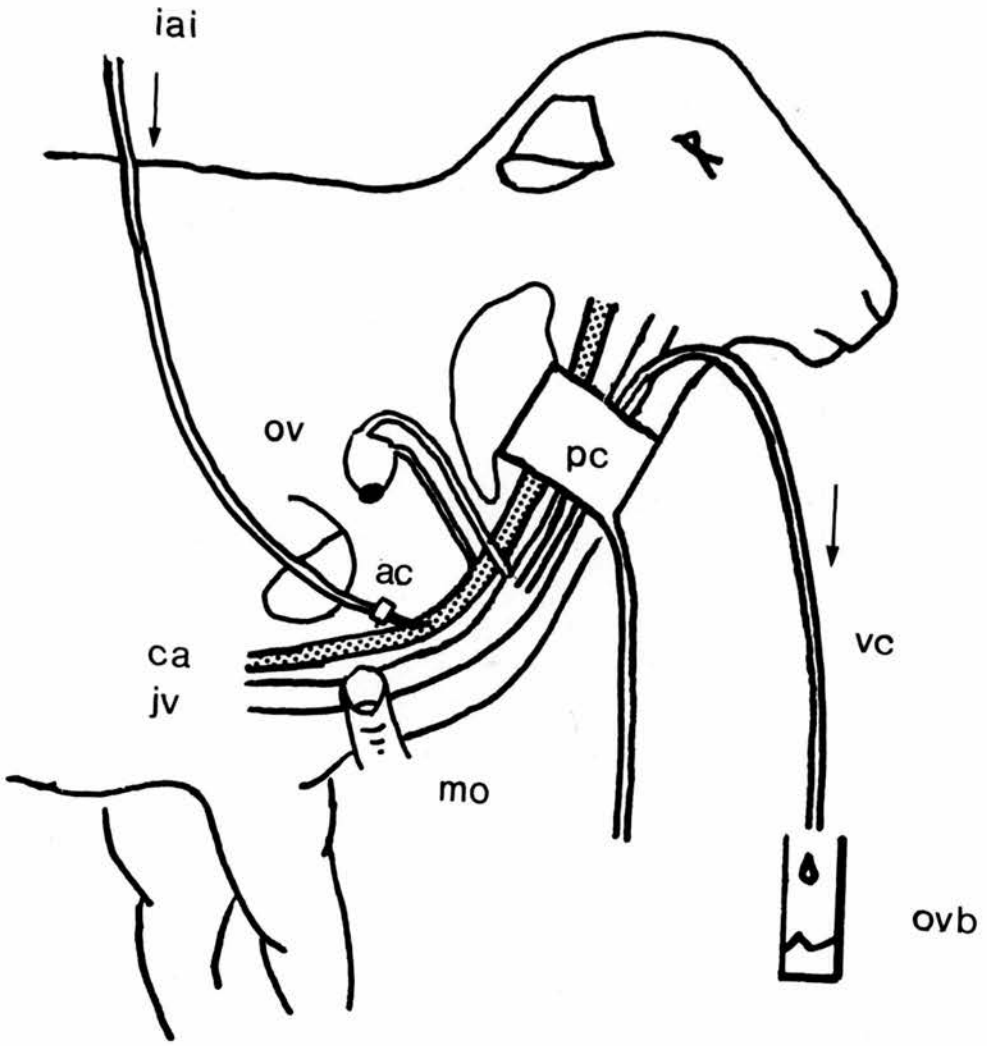
Testing the ewes for oestrus

This was done by turning an experienced ram into the same pen as the ewe. The behaviour of the two sheep was studied for 10 minutes from behind a window. The ewe was in oestrus if she stood for the ram and allowed him to mate. During pro-oestrus, the ewe allows the ram to tease her but does not stand and he is unable to mate.

Intra-arterial infusions

On the morning of experiments, the jugular vein and the carotid artery anastomosed to the blood vessels of the transplanted ovary were canmulated as described previously ( McCracken, Uno, Goding, Ichikawa & Baird, 1969 ). The contralateral jugular vein was also cannulated with a number 2 Braunula ( Armour, Eastbourne, Sussex ). The Braunula was later connected to a blood administration set ( Avon Rubber, Birmingham ). Prophylactic antibiotic therapy was given ( 4 ml i.m. 'Streptopen', Glaxo ).

After cannulation, the ewe was placed into a metabolism cage. One hour later, the ewe was heparinized with an intravenous injection of heparin ( Weddel, London, 5000 units ). Two hours after cannulation, control periods of infusions were started. Infusions into the carotid artery were maintained by the use of a constant infusion pump ( Harvard Infusion Apparatus, Millis, Massachusetts, U.S.A. ).


iai
ov
pc
ac
ca
jv
vc
mo
ovb

Legend to figure 11

Diagram to show intra-arterial infusion of solutions and collection of ovarian vein blood from ewes with transplanted ovaries

| | |
|---|---|
| ca | carotid artery |
| jv | jugular vein |
| ov | ovary |
| pc | pneumatic cuff |
| vc | venous catheter |
| ac | arterial catheter |
| mo | manual occlusion of the jugular vein |
| iai | intra-arterial infusion |
| ovb | ovarian vein blood |

A sphygmomanometer cuff ('Velcro for infants', A.C. Crosser, London ) was placed around the skin loop cranial to the bridge containing the ovary. The cuff was inflated to 200 mm/Hg to occlude the arterial blood flow and to force that vessel's blood to perfuse the ovary.

Ovarian vein blood was collected by free fall from the jugular catheter after manual occlusion of the jugular vein at a point caudal to its anastomosis with the ovarian vein. The first 5 ml blood was not collected for steroid determination, but it was later transfused back into the ewe. A foot-operated stop-watch was used for timing 25 ml blood collections into a sterile heparinized container. After each blood collection, the catheter was flushed with saline containing 10 U/ml heparin and closed by meass of a two way tap.

A 2 ml aliquot was removed from some blood samples, stored on ice, and later used for determining the haematocrit ( Whitby & Britton, 1957 ). The remainder of each sample was centrifuged in a refrigerated machine at 6°C. A 7 ml aliquot of plasma was removed from each sample with a sterile pipette. The plasma samples were then stored in a deep freeze until they were assayed. The red cells were resuspended in sterile saline and transfused into the ewe to maintain the animal's blood volume.

## CALCULATIONS

### Calculation of plasma flow

Plasma flow was calculated from the observed blood flow and a knowledge of the haematocrit. During infusions, samples of ovarian vein blood were removed for determining the haematocrit at half hourly time intervals. The haematocrits seldom varied by more than 2 % in half an hour. The haematocrits of samples taken during the intervening period of time were estimated by interpolation from the two measured samples.

### Calculation of secretion rate

In calculating the secretion rate, it was assumed that peripheral progesterone concentrations were negligible in comparison with those in the ovarian vein plasma. This assumption was verified for each experiment. It was also assumed that all the secreted hormone was found in the plasma. When tritiated progesterone was added to whole sheep blood, 89.8 $\pm$ 0.75 ( S.E.M.) %, n = 12, of the equilibrated radioactivity was found in the plasma after centrifugation. Peripheral jugular and ovarian vein blood behaved in the same fashion. The addition of as much as 10 $\mu$g/ml progesterone to whole blood failed to alter the distribution of added radioactivity between plasma and red cells. It was concluded that this assumption leads to a constant underestimation of ovarian progesterone secretion rate. Similar tests with oestradiol-17$\beta$ were not carried out. The secretion rates quoted have not been corrected to take account of the steroid bound to red cells.

Statistical tests

Tests for significance between groups of values were conducted by standard methods described by Snedecor ( 1956 ). Trends were examined for significance by least squares, linear regression analysis. Trends were compared by analyses of covariance. Tables and formulae published in 'Documenta Geigy' ( Diem, 1962 ) were used extensively. Calculations were performed with an Olivetti 'Programma 101' desk top computer. Programmes were obtained from the Olivetti Software Library or were written by the author.

Comparisons between values obtained in two different periods of an experiment may be made by a Student's 't' test. However, some responses are of shorter duration than the experimental periods and the significance of short term responses is obscured. Two approaches were used in dealing with this difficulty; both were used in chapter 3.

In the first approach, the standard deviation of values observed in a preceding control period was calculated, and limits were set at two standard deviations on either side of the mean. If a random and normal distribution is assumed, then the probability of finding values outside these limits is about 0.05. This assumption was made and the limits are referred to as the '95 % control limits'. If more than 2 out of 6 or 3 out of 12 values lay outside of these limits on one side only, then it was judged that a significant change had occurred. The disadvantage of this approach is that it is not true if there is a tendency for values to become higher or lower with the passage of time.

In the second approach, values from different infusions on different animals were divided into discrete time intervals. Values for the same time interval were combined and compared with control values. Control blood flow, and especially progesterone secretion rate varied greatly between ewes. Thus values from different ewes could be compared only after each had been expressed as a percentage of the mean control period value for its own control period. Here it is assumed that animals give the same pattern of response. However, this technique has the advantage of smoothing out variation found in individual experiments.

It is concluded that there is no really satisfactory method for analysing dynamic responses, unless very numerous observations have been taken.

## ASSESSMENT OF THE OVARY TRANSPLANT

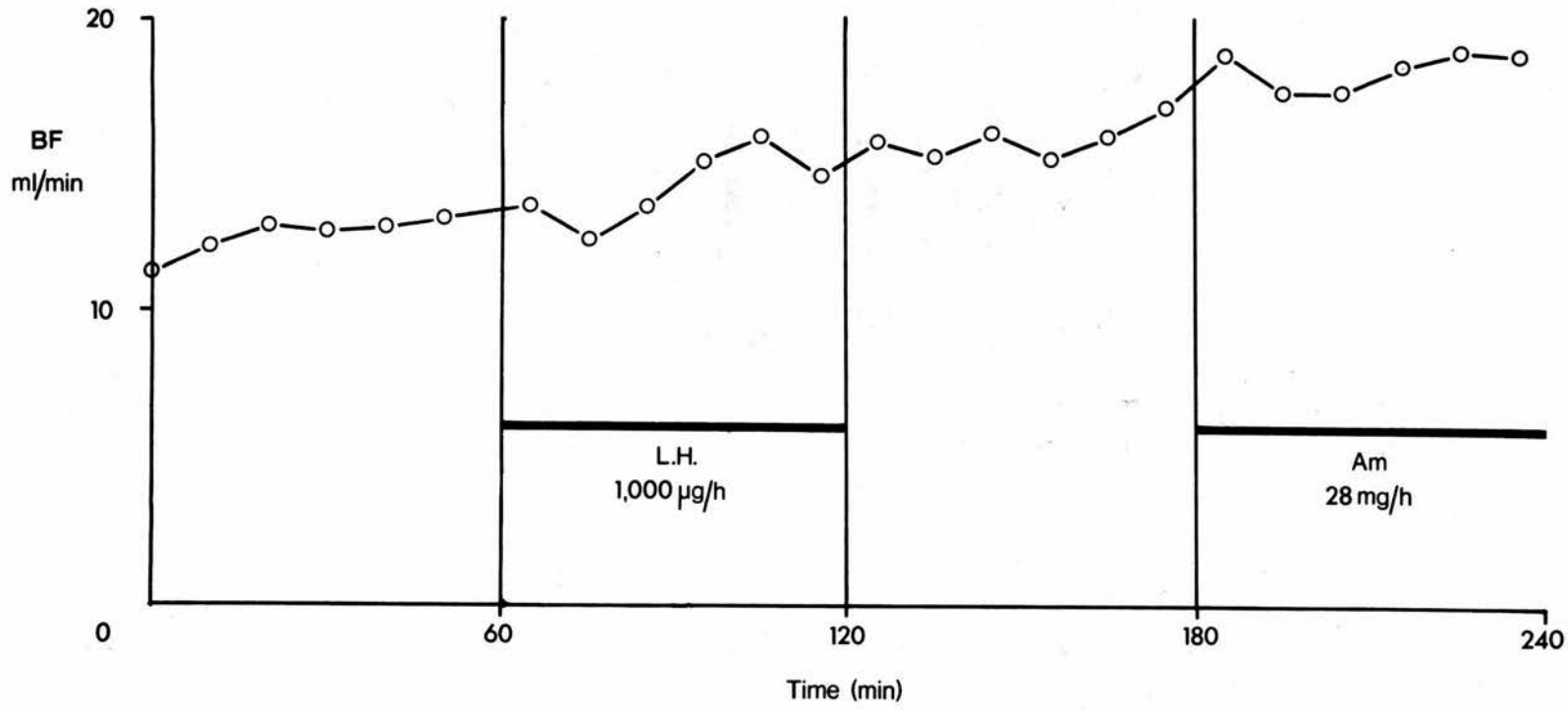
Two months after the transplant operations had been performed, each ewe was cannulated and ovarian vein blood was collected. The progesterone concentration in these samples was assayed. Two ( CB94 and D305,) out of seven ewes were found not to be secreting progesterone. These two ewes were treated with systemic gonadotrophin ( 750 I.U. PMSG i.m.i.,'Folligon', Intervet, followed by 5000 I.U. HCG i.v.,'Pregnyl', Organon, two days later). The ovaries of both ewes secreted progesterone after this treatment.

Table 5.

Blood flow (BF) and progesterone secretion rate (SR) from the ovary *in situ*

| Ewe No. | Day of cycle | Structures on the ovary of the cannulated side | BF* ml / min | SR* µg / min |
|---|---|---|---|---|
| 91-814 | 8 | 1 *corpus luteum* and several small follicles. | 5.6 ± 0.51 (3) | 2.2 ± 0.06 (3) |
| 91-890 | 8 | 1 *corpus luteum* and 1 follicle 0.5 cm diameter. | 11.2 ± 0.20 (4) | 2.0 ± 0.07 (4) |
| 91-870 | 8 | 10 follicles 0.5 cm in diameter. | 5.7 ± 0.25 (4) | 0.0 ± 0.00 (4) |
| 91-920 | 15 | 2 *corpora lutea*. | 8.6 ± 0.43 (4) | 3.2 ± 0.16 (4) |
| 91-926 | 15 | 1 follicle 0.5 cm in diameter. | 2.7 ± 0.01 (3) | 0.0 ± 0.00 (4) |
| 91-634 | 15 | 2 follicles 0.5 cm in diameter and 1 *corpus luteum*. | 11.1 ± 0.42 (4) | 2.7 ± 0.11 (4) |

* Mean ± standard error ( number of determinations )


20
BF
ml/min
10
0
60
120
180
240
Time (min)
L.H.
1,000 µg/h
Am
28 mg/h

Legend to figure 12

Changes in blood flow (BF) from the skin loop during intra-arterial infusion of luteinizing hormone (LH) and aminophylline (Am).

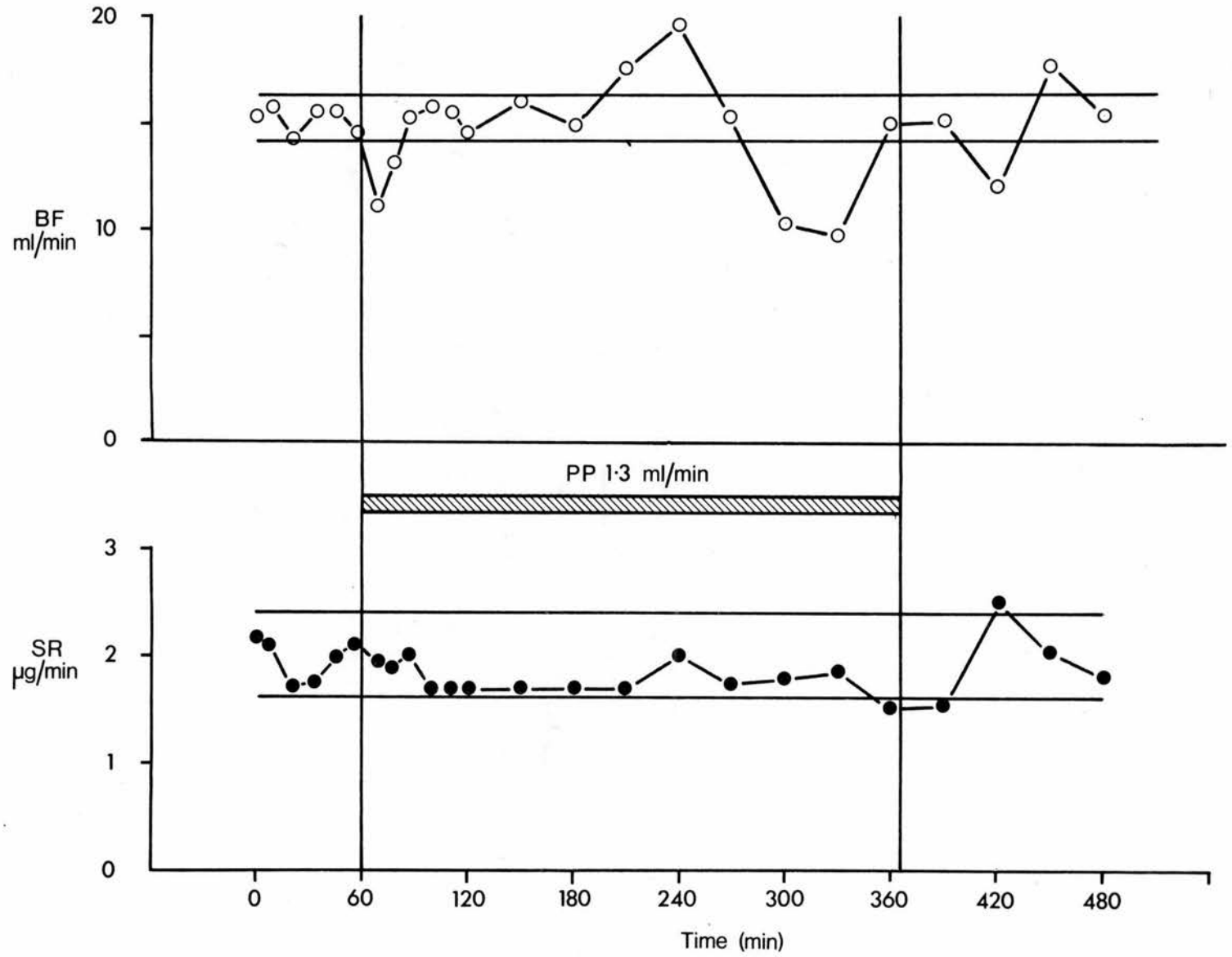
Experiment A; D442, 20-3-73. An attempt to transplant part of the uterus and the vagina to the loop of this animal had failed. The solutions were infused at the rate of 11.3 ml / h. Saline was infused during the control periods. Luteinizing hormone was NIH-LH-S17 ( see page 114 ). Aminophylline is a phosphodiesterase inhibitor ( see page 129 ).

Radioangiographic studies by Dr. D.T. Baird on D342 and D304 showed that both ewes have a patent ovarian circulation through the anastomosed blood vessels.

The blood flows and progesterone secretion rates from ewes with transplanted ovaries can be compared with a few results obtained by Dr. D.T. Baird on ewes at operation under 'Nembutal' induced halothane maintained anaesthesia ( see table 5 ). It is apparent that the corpus luteum is the only ovarian structure in the ewe capable of secreting progesterone. The observation that two corpora lutea secrete more progesterone than one is in line with previous reports ( Short, 1964; Thorburn, Bassett & Smith, 1969 ).

The ovarian blood flows measured in situ are much lower than those generally found from the autotransplanted ovary. Part of the difference is made up by blood which comes from the skin of the loop and the bridge containing the transplanted ovary. The blood flow from the skin was measured by Dr. D.T. Baird in 11 ewes which had not yet received transplanted organs. The mean was 5.1 $\pm$ 0.7 ( S.E.M. ) ml/min. As the other ovary is removed from those ewes bearing autotransplanted ovaries, the transplanted organ would be expected to undergo hypertrophy - the normal consequence of hemicastration ( Sundaram & Stob, 1967 ).

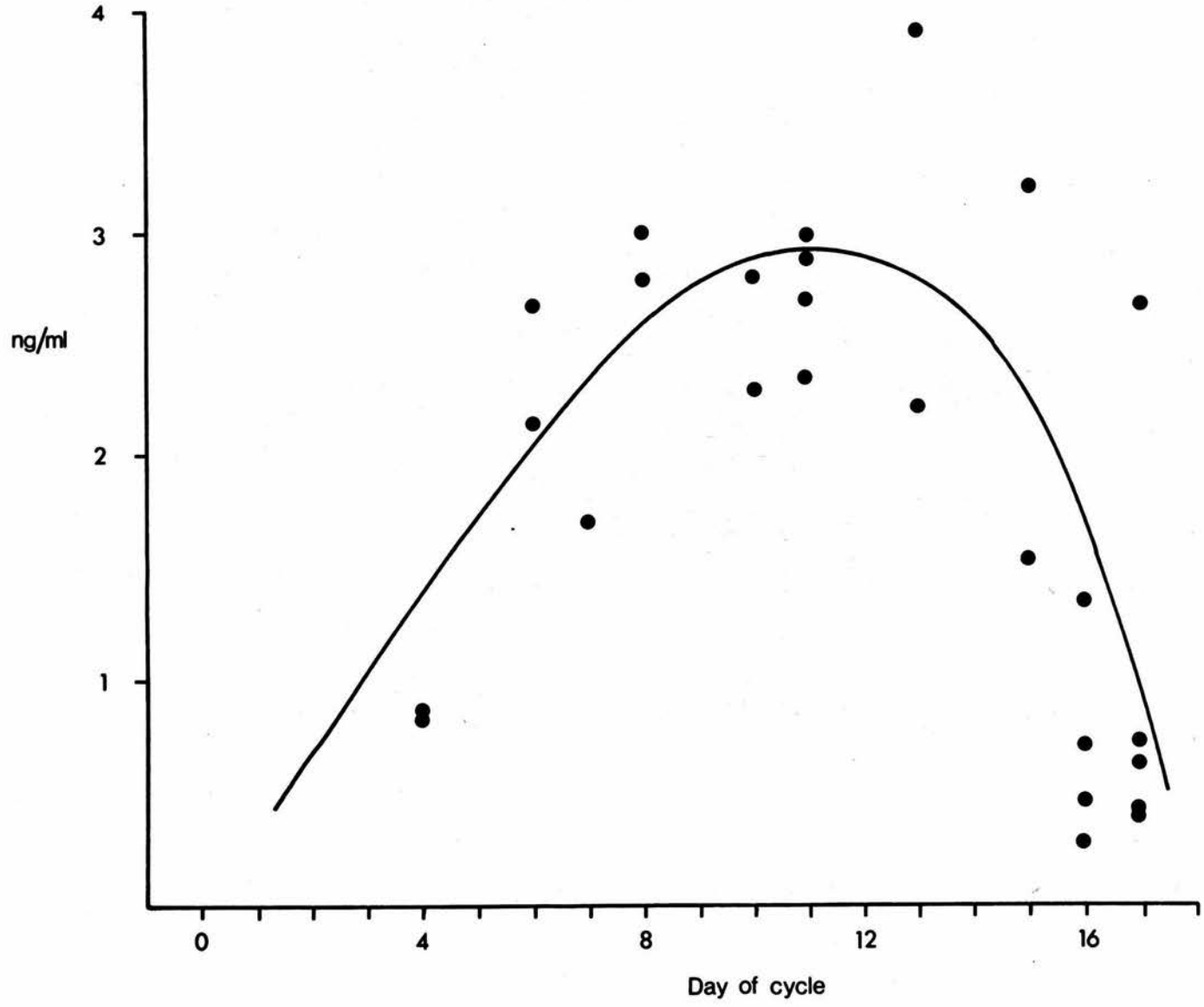
Figure 12 shows the changes in blood flow resulting from infusions of LH and aminophylline into a ewe bearing a skin loop where a uterine autograft had failed. It is likely that the extra bulk of wound tissue is responsible for the high blood flow seen in


20
10
0
BF
ml/min
PP 1·3 ml/min
3
2
1
0
SR
µg/min
0
60
120
180
240
300
360
420
480
Time (min)

Legend to figure 13

Failure of peripheral plasma infusion to affect progesterone secretion.

Experiment B: D305, 1-4-71. Peripheral plasma was obtained from blood freshly drawn from the jugular vein of a Finn-Dorset ewe that had just entered summer anoestrum. Plasma was infused into the transplanted ovary at a rate of 1.3 ml / min. The figure shows changes in blood flow (BF) and progesterone secretion rate (SR). The duration of the infusion of peripheral plasma (PP) is indicated by the horizontal bar. The horizontal lines indicate the 95 % conrol limits for values observed during the first hour of the experiment.


4
3
2
1
ng/ml
0
4
8
12
16
Day of cycle

Legend to figure 14

Variation in the peripheral plasma progesterone concentration throughout the oestrous cycle of intact ewes

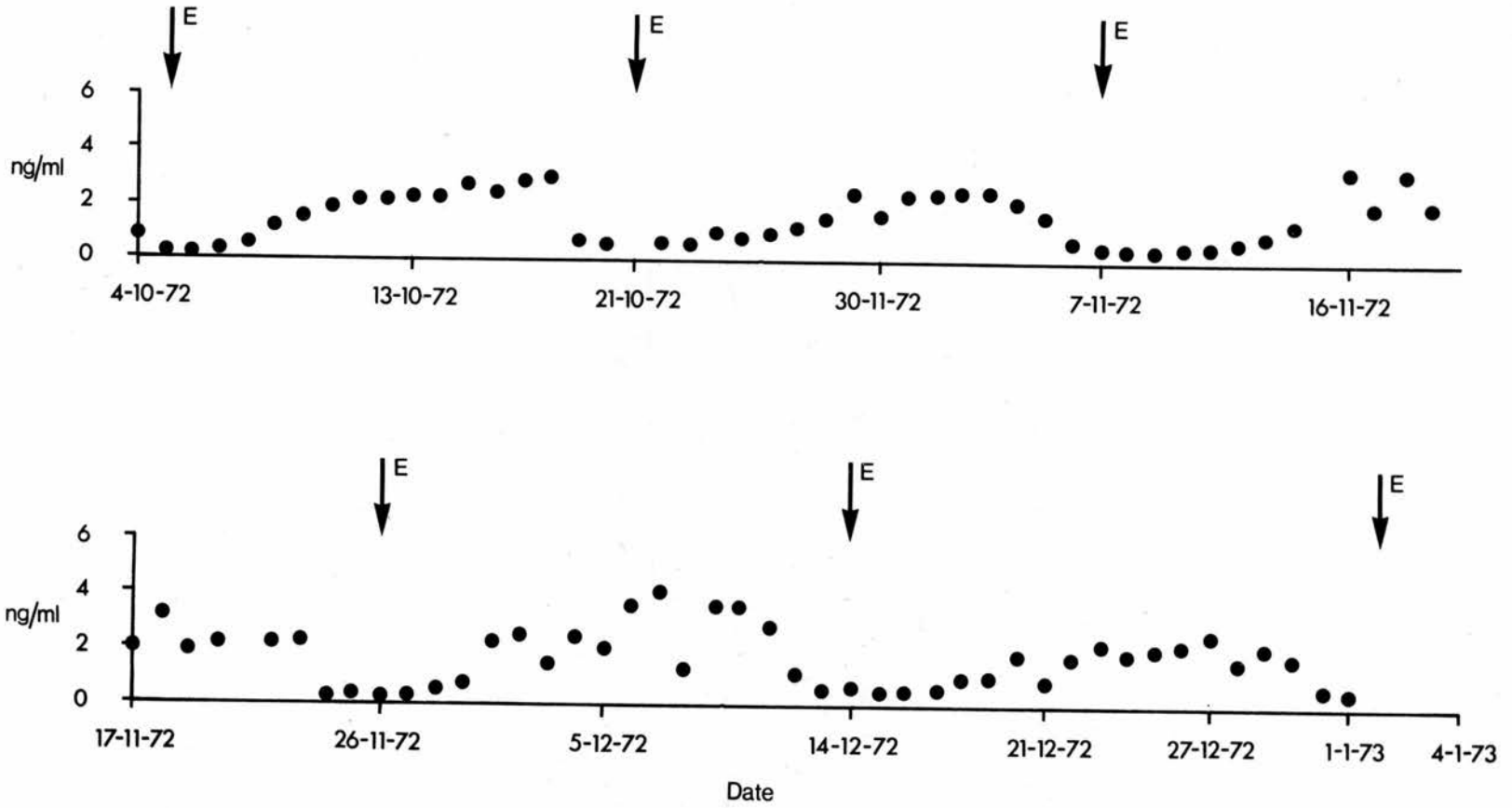
Blood was drawn from the jugular vein of Finn-Dorset ewes by Dr. R.B. Land. Oestrus was determined by running the ewes with a vasectomized ram marked with a marking crayon. The day of onset of oestrus is designated day 0.

this experiment. The blood flow rose steadily throughout the experiment, and it is possible that this result occurred because room temperature increased, and the peripheral capillaries became more dilated. It is concluded from this experiment that the rate of blood flow is not a reliable indication of ovarian blood flow in the autotransplant preparation.

Figure 13 shows the changes in blood flow and progesterone secretion rate observed during an infusion of 1.3 ml/min peripheral plasma into the transplanted ovary. Although blood flow from the ovary became more variable during plasma infusion, the secretion rate remained within the 95 % control limits until the end of the infusion period. It was concluded that the infusion of non-specific proteins at a high dose rate had little effect on ovarian progesterone secretion rate.

The variation in progesterone concentrations in peripheral plasma during the oestrous cycle of the ewe have already been described ( see chapter 1 ). That pattern was confirmed in a small series of samples collected by Dr. R.B. Land from normal Finn-Dorset ewes at known stages of the oestrous cycle ( see figure 14 ).

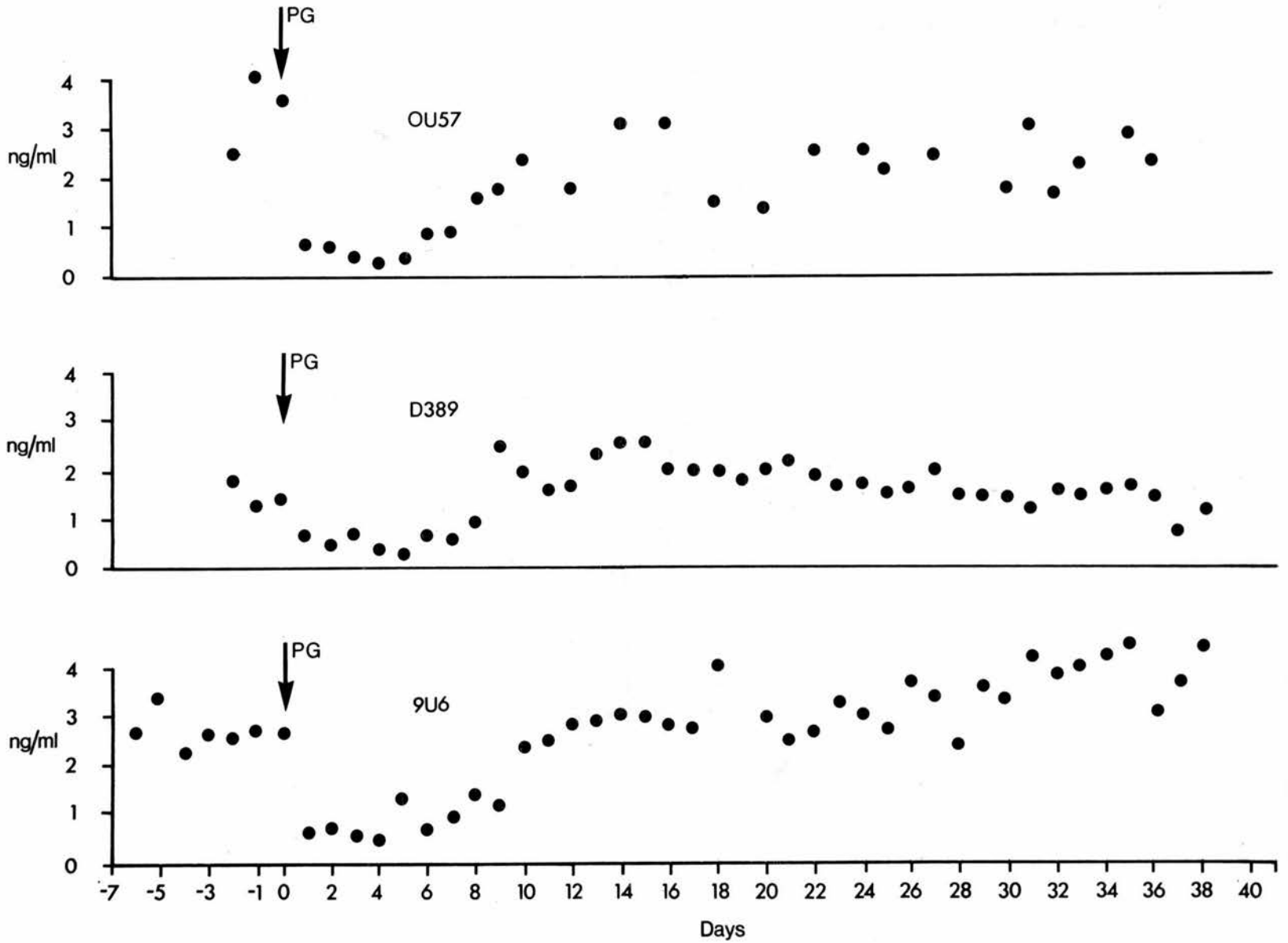
No such cyclical variation in peripheral progesterone concentrations was noted in daily samples collected by Mr. A.G. Wheeler from three ewes with ovarian transplants ( see figure 16 ). The ewes with transplanted ovaries also fail to show periods of heat during the breeding season. These observations confirm a previous report ( Baird, Goding, Ichikawa & McCracken, 1968 ). The ewes with


E
E
E
6
4
2
0
ng/ml
4-10-72
13-10-72
21-10-72
30-11-72
7-11-72
16-11-72
E
E
E
6
4
2
0
ng/ml
17-11-72
26-11-72
5-12-72
14-12-72
21-12-72
27-12-72
1-1-73
4-1-73
Date

Legend to figure 15

Variation in the peripheral plasma progesterone concentration of a ewe bearing a transplanted uterus and ovary.

The left ovary, left uterine horn and the vagina of MX16 were transplanted with vascular reanastomoses to a prepared skin loop. The vascular connexions between the uterus and the ovary are retained in this preparation. The transplant operation was performed by Drs. Baird, Land and Scaramuzzi on 17-8-72. 'E' indicates days of onset of oestrus.


PG
OU57
ng/ml
4
3
2
1
0
PG
D389
ng/ml
4
3
2
1
0
PG
9U6
ng/ml
4
3
2
1
0
-7 -5 -3 -1 0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40
Days

Legend to figure 16

Maintained luteal function in three ewes bearing transplanted ovaries

Dr. D.T. Baird and Mr. A.G. Wheeler regressed maintained corpora lutea by means of intra-arterial infusions of prostaglandin $F_{2\alpha}$ ( see chapter 6 ). 'PG' indicates the days of prostaglandin infusion. Daily blood samples were collected by Mr. A.G. Wheeler.

transplanted ovaries have maintained corpora lutea characteristic of hysterectomized ewes ( Wiltbank & Casida, 1956 ). This condition in the ewes with transplanted ovaries is likely to be caused by the physical separation of the ovary from the uterus ( Harrison, Heap & Linzell, 1968 ). One ewe bearing an ovary transplanted with its connexions to the ipsilateral horn intact by simultaneous transplantation of part of the uterus and the vagina ( McCracken, Glew & Levy, 1970 ) showed regular periods of heat. Analysis of peripheral plasma progesterone concentrations in samples taken daily revealed cyclical changes characteristic of normal ewes ( see figure 15 ). The discharge of mucus from the uterus, and the changes in the appearance of the vagina correspond to previous observations on intact ewes ( Grant, 1934; Marshall, 1903 ). In this study, collections of blood, observations of the uterus and testing for heat were undertaken by Dr. D.T. Baird, Dr. R.J. Scaramuzzi, Dr. R.B. Land and Mr. A.G. Wheeler.

Other aspects of ovarian function in this preparation have been reviewed ( Goding, Baird, Cumming & McCracken, 1972 ). It was concluded that the histology of the ovary was normal and that the secretion of the major ovarian steroids was similar to that seen in situ. However, the absence of the normal luteolytic stimulus in the ovary transplant ewes causes them to show prolonged luteal function characteristic of hysterectomized ewes.

CHAPTER THREE

# THE EFFECTS OF LUTEINIZING HORMONE

## Introduction

One of the tests for luteotrophic activity is to find whether a hormone preparation is able to stimulate progesterone secretion from luteal tissue. In the sheep ovary, the corpus luteum alone secretes progesterone. It is therefore possible in this animal to test for luteotrophic activity by observing the effect of hormones on ovarian progesterone secretion rate.

The addition of LH to in vitro incubations of slices of sheep luteal tissue increases progesterone synthesis ( Kaltenbach, Cook, Niswender, Norton & Nalbandov, 1967 ). However, experiments in vitro may not give a true indication of what happens in vivo.

At operation under anaesthesia, two groups have found LH to increase ovarian progesterone secretion rate in the ewe ( Domański, Skarzeczkowski, Stupnicka, Fitko & Dobrowolski, 1967; Cook, Kaltenbach, Niswender, Norton & Nalbandov, 1969 ) while two other groups were unable to find an increase ( Short, McDonald & Rowson, 1963; Hixon & Clegg, 1969 ). Furthermore, Cook's group claimed that the data presented by Domański's group did not lead to the conclusion

Table 6.

Summary of preliminary experiments involving LH infusion

| Expt. No. | Ewe No. | Date | Dose rate µg / min | Duration min | Prior infusions on the same day | Previous experiments | Comments |
|---|---|---|---|---|---|---|---|
| C | CB69 | 16-5-69 | 1 | 60 | none | none | - |
| | | | 10 | 60 | 1 µg LH | none | - |
| D | D342 | 11-3-70 | 100 | 30 | 14.5 mg cAMP | none | see chapter 7. |
| E | D342 | 25-3-70 | 100 | 30 | none | expt. D | see chapter 7. |
| F | D304 | 16-4-70 | 10 | 60 | none | none | see also table 7, expt. 1. |
| | | | 100 | 60 | 10 µg LH | none | |

Table 7.

Summary of experiments used for determining the pattern of the response to LH

| Expt. No. | Ewe No. | Date | Dose rate μg / h | Prior infusions on the same day | Previous experiments | Comments |
|---|---|---|---|---|---|---|
| 1 | D304 | 16-4-70 | 1000 | 0.1 mg LH | none | see table 6 |
| 2 | CB69 | 23-4-70 | 1000 | none | expt. C | see table 6 |
| 3 | D342 | 30-4-70 | 800 | none | expt. E | see table 6 |
| 4 | CB94 | 28-5-70 | 1000 | none | PMSG & HCG Dec. 1969 | - |
| 5 | D342 | 2-7-70 | 1000 | 1.8 mg dbcAMP | expt. 3 | see also chapter 7 |
| 6 | D342 | 10-9-70 | 1000 | 50 mg dbcAMP | expt. 5 | see also chapter 7 |
| 7 | D305 | 24-9-70 | 1000 | none | PMSG & HCG May 1970 | see also chapter 4 |
| 8 | D342 | 29-10-70 | 1000 | none | expt. 6 | see also chapter 4 |
| 9 | D342 | 12-11-70 | 1000 | none | expt. 8 | see also chapter 4 |

that LH significantly increased progesterone secretion. An anaesthetic agent was found to depress progesterone secretion and ovarian blood flow ( Hixon & Clegg, 1969 ). The amounts of LH administered were very large, and all except Domański's group gave the hormone systemically and indirect effects mediated by the pituitary could not be ruled out.

The first study of the effect of LH on ovarian steroid secretion in the conscious sheep was done using the ovarian transplant preparation ( McCracken, Uno, Goding, Ichikawa & Baird, 1969 ). They found that physiological amounts of LH increased ovarian steroid secretion, but it must be admitted that the number of control samples taken was limited. In most of their infusions, the sheep had been previously treated with PMSG and HCG in order that many corpora lutea secreting much progesterone should be formed.

It was hoped to confirm the results obtained by workers at the Worcester Foundation, and to show that the same effect could be obtained with ovaries where superovulation had not been induced. It was also hoped to use the transplanted ovary as an in vivo perfusion apparatus to study the pattern of the ovarian response to LH.

Materials and methods

Six ewes bearing autotransplanted ovaries were used in this study ( see tables 6 and 7 ). Two ewes ( CB94 and D305 ) were found not to be secreting appreciable amounts of progesterone after transplantation and they were therefore treated with PMSG and HCG.

Assessment of the state of the ovaries which were infused is largely speculative, but it may be inferred that they all contained luteal tissue because progesterone was being secreted. In two experiments where the secretion rate was low ( 1 and 4 ), the ovaries probably contained 'moribund' long-maintained corpora lutea. In the case of experiment 4, the observed secretion is likely to have been from several 'dying' corpora lutea because CB94 had been superovulated six months previously. The high secretion rate from D305 in experiment 7 indicates that multiple corpora lutea had been maintained during anoestrum from the date of their formation after superovulation had been induced at the end of the previous breeding season. In the remaining experiments, it would be reasonable to assume that one or two corpora lutea formed after natural ovulation were responsible for progesterone secretion.

The ewes were prepared by Dr. D.T. Baird for intra-arterial infusions and for collections of ovarian vein blood as described in chapter 2. The progesterone concentration in ovarian vein plasma samples was estimated as described previously. The estimates were corrected for the individually determined procedural losses. The progesterone secretion rates were calculated.

The experiments are summarized in tables 6 and 7. During each experiment, more than one infusion was conducted, Each experimental infusion period was preceded by a control period throughout which saline was infused at a rate of 17.3 ml/h. During experimental periods, solutions of gonadotrophins were infused, also at the rate of 17.3 ml/h. Ovarian vein blood collections were taken

by Dr. D.T. Baird every 10 minutes or more frequently during experimental and control periods. In the series of experiments listed in table 7, a control period lasting one hour was followed by a period of LH infusion also lasting one hour.

Ovine luteinizing hormone ( LH ) was a gift from the National Institute of Health, Bethesda, Maryland, U.S.A.. According to the institute, the batch used ( NIH-LH-S14, ) had a mean relative potency of 0.98 NIH-LH-S1 units / mg using the rat ovarian ascorbic acid depletion test. The preparation is said to be contaminated with less than 0.17 U.S.P. units/mg thyroid stimulating hormone, and with 0.023 NIH-FSH-S1 units/mg follicle stimulating hormone.

On the day before an experiment, LH was weighed and dissolved in 0.9 % sterile saline ( Baxter Laboratories, Thetford, Norfolk ). The solution was sterilized by passage through an ultrafilter ( SXGS 025 0S; Millipore, Bedford, Massachusetts, U.S.A. ), and stored overnight at $4^{o}$C.

After these experiments had been completed, it was discovered that a good part of LH in solution was absorbed by the ultrafilters used for sterilization. Dr. W. Carr ( Animal Breeding Research Organization, Edinburgh ) kindly carried out LH radioimmunoassays for me. It was found that a single passage of an LH solution through such a filter removed 61 $\pm$ 1.6 ( S.D.) % ( n = 4 ) of the immunological potency. This result was confirmed by a single bioassay result ( rat ovarian ascorbic acid depletion test ) obtained on an infusion solution by Dr. D.B. Crichton ( Sutton Bonnington ).

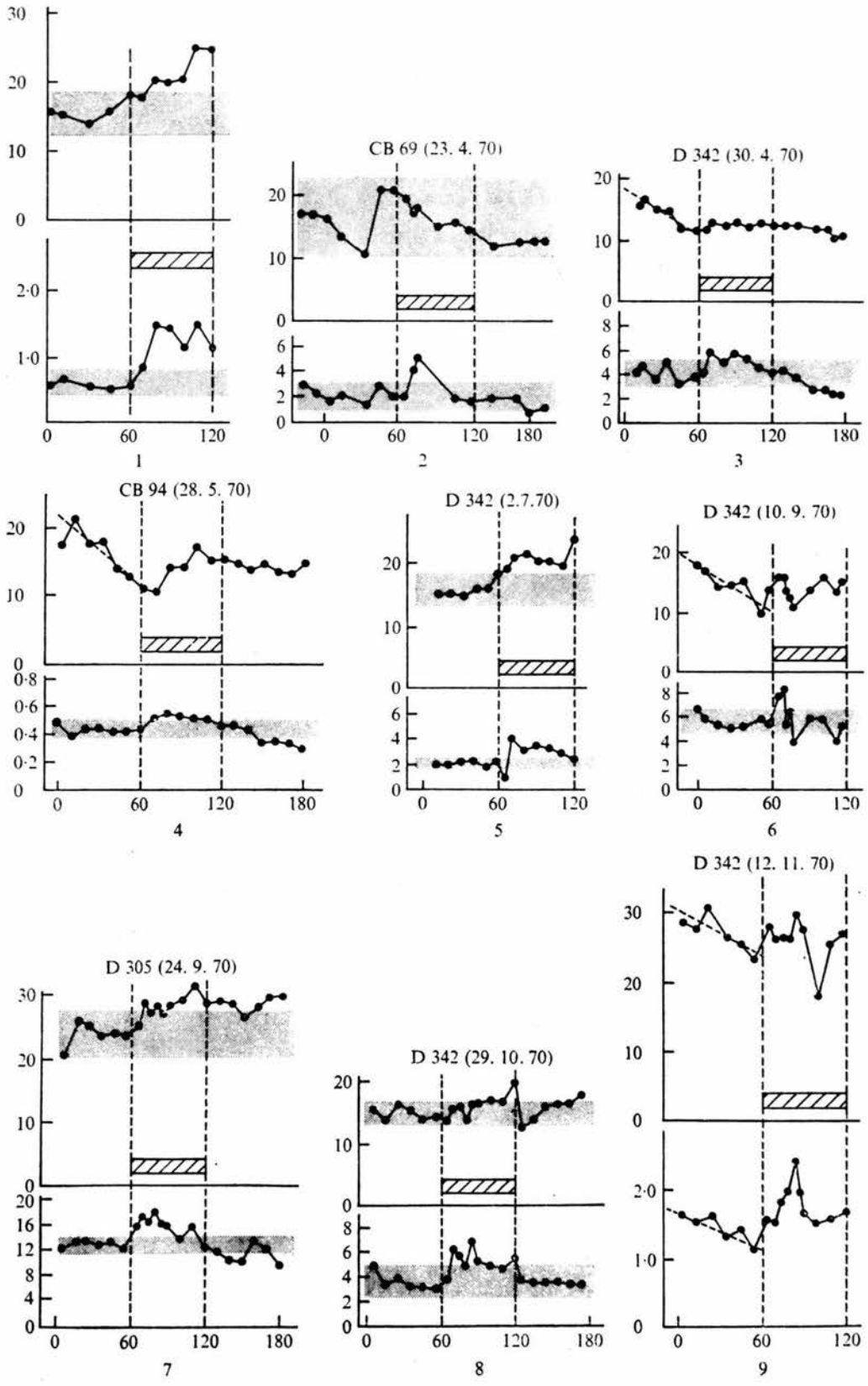
It showed that 64 % of the biological potency had been removed by sterilization.

In order that it might be possible to compare the patterns of responses obtained during different experiments, the values for blood flow or for secretion rate were expressed as a percentage of the mean value observed during the previous control period. This was necessary because blood flow and especially secretion rate varied greatly between experiments. Individual values were placed into five- or ten-minute time segments and the mean relative value for each time interval was calculated.

Results

In four preliminary experiments, LH was infused at nominal dose rates of between 1 and 100 μg/h (see table 6 ). The infusion of 1 or 10 μg/h in experiments C and F failed to increase either blood flow or secretion rate significantly. In experiment F, 100 μg/h infusion increased progesterone secretion transiently. In experiment E blood flow increased to double the control levels during 100 μg/h LH infusion, whereas secretion rate was unaffected. In the experiments used for characterizing the pattern of the response to LH, a much higher dose rate was employed because these preliminary experiments showed that a consistent effect was not always produced by the lower dose rates.

The changes in blood flow and secretion rate for nine individual experiments where high doses of LH were infused, are shown


CB 69 (23. 4. 70)
D 342 (30. 4. 70)
CB 94 (28. 5. 70)
D 342 (2.7.70)
D 342 (10. 9. 70)
D 305 (24. 9. 70)
D 342 (29. 10. 70)
D 342 (12. 11. 70)
1
2
3
4
5
6
7
8
9

Legend to figure 17

The response to intra-arterial luteinizing hormone infusion in nine experiments

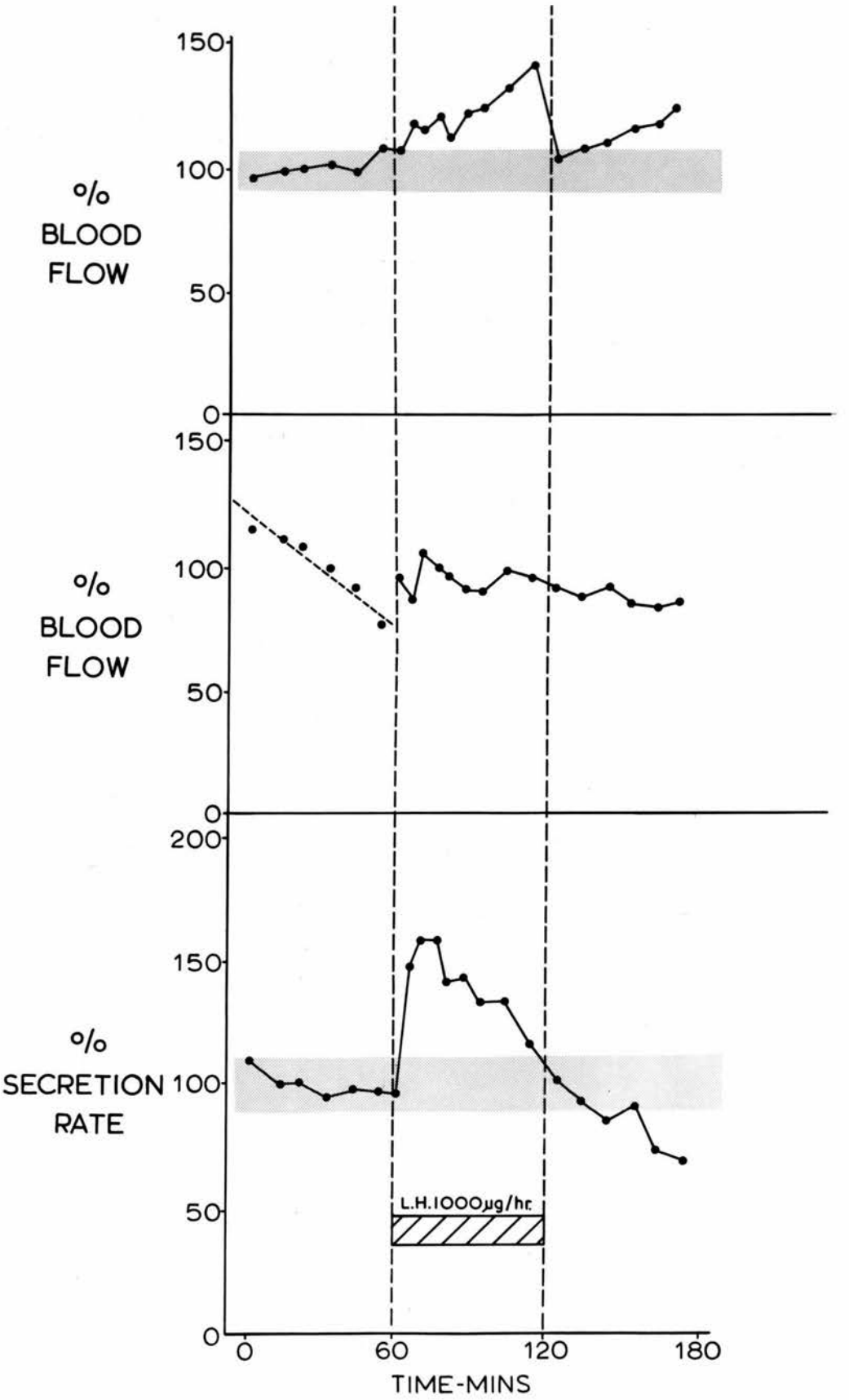
In the top half of each sub-figure the changes in blood flow ( ml / min, ) and in the lower half of each sub-figure the changes in progesterone secretion rate ( $\mu g$ / min,) are illustrated. The horizontal axes indicate the time in minutes. The stippled bands indicate the 95 % control limits for values observed during the first hour of each experiment. Dashed lines represent significant ( $P<0.05$) linear regression equations. The number of each experiment, the identity of the ewe and the date are given for each infusion.

in figure 17. From the results of these nine experiments, the mean pattern of the response to a continuous LH infusion was calculated ( see figure 18 ).

The mean secretion rates observed during control periods varied: the lowest was 0.4 μg/min ( in experiment 4 ); the highest was 12 μg/min ( in experiment 7 ). The mean control blood flows were generally about 15 ml/min. In two experiments ( 7 and 9 ), the flow was rather higher.

In terms of the individual experiments, secretion rate was fairly constant during control periods, except in experiment 9 where a significant downward trend occurred. LH infusion in every case led to an increase in progesterone secretion so that at least two values were above the control limits. However, the rise was transient, and the peak values were generally seen within 20 minutes of the start of an LH infusion. Because the higher values were not usually sustained, the mean secretion rate was significantly higher ( $P < 0.05$ ) than that observed in the preceding control period in only five experiments ( 1, 4, 7, 8 and 9 ). After the early peak in secretion rate, values generally fell, and they continued to fall in the following hour. In three of the four experiments where blood sampling was continued, the final secretion rates were below the lower control limits.

Control blood flow values were rather less steady than those for progesterone secretion rate. In four experiments ( 3, 4, 6 and 9 ), the blood flow fell progressively and the trends were sig-


150
100
50
0
% BLOOD FLOW
150
100
50
0
% BLOOD FLOW
200
150
100
50
0
% SECRETION RATE
L.H.1000µg/hr.
0
60
120
180
TIME-MINS

Legend to figure 18

Pattern of the responses to luteinizing hormone infusion

The stippled bands indicate the 95 % control limits for values observed during the control period. The dashed line represents a significant, linear regression equation.

a), mean relative blood flows in experiments 1, 5, 7 and 8.

b), mean relative blood flows in experiments 3, 4, 6 and 9.

c), mean relative progesterone secretion rate in all nine experiments.

nificant ( $P<0.05$ ). In these experiments, LH either halted the decline ( experiments: 3, 6 and 9 ), or reversed the trend ( experiment 4 ), In experiment 2, control blood flow was variable, and no trend was established. In the remaining experiments ( 1, 5, 7 and 8 ), control blood flow was steady, and LH caused a progressive rise in values. The highest flows were observed at the end of the period of LH infusion.

Analysis of covariance showed that it was possible to represent control period secretion rate trends from individual experiments by a common regression line ( regression coefficient = -0.28 % per min, 44 degrees of freedom, $P<0.05$ ). However, it was not possible to analyse the responses to LH in this manner because the pattern was non-linear ( see figure 18c ).

It became apparant that individual regression equations for control blood flow trends could not be validly represented by a common equation. However, if experiment 2 was excluded on account of the large residual variance about the regression line, the remaining eight experiments could be divided into two groups according to the trend in control blood flow. Where there was a decline ( experiments 3, 4, 6 and 9 ), LH halted this trend ( see figure 18b ). When the control blood flow was steady ( experiments 1, 5, 7 and 8 ), LH caused a steady rise ( see figure 18a ). In neither case was analysis of variance helpful in investigating the responses because the means from individual experiments were significantly different.

The different patterns of control blood flows did not affect either the pattern or the magnitude of the secretion rate responses to LH significantly.

Discussion

Absorption of LH by 'Millipore' filters was discovered after these experiments had been completed. However, the proportion of the total LH removed by sterilization appears from the immunoassay data to be reasonably constant. A nominal infusion rate of 1000 μg/h is likely to correspond to an actual infusion rate of 400 μg/h.

LH absorption by ultra-filters has been noted in discussion ( Eshkol, 1969 ), and HCG is similarly affected ( Dr. B.M. Hobson, Hormone laboratory, Edinburgh University, unpublished ). Perhaps these results should have been anticipated; the makers describe their filters as comprising 'mixed esters of cellulose', and cellulose column or paper chromatography is a technique used for purifying gonadotrophins.

Many earlier workers remarked upon the power of non-specific substances to increase the biological activity of pituitary extracts ( Hellbaum, 1936; Saunders & Cole, 1936; McShan & Meyer, 1941 ). Possibly the effect of these substances is to prevent gonadotrophin from sticking to surfaces of containers.

The inconsistent effects of LH infusion when dose rates below 1000 µg/h were employed conflict with previous findings ( McCracken, Uno, Goding, Ichikawa & Baird, 1969 ). However, it is possible that my results were caused by a failure to deliver LH to the ovary. This matter is discussed further in chapter 6 which reports experiments where the correct dose of LH was delivered.

It is likely that the concentration of LH in the blood perfusing the ovary in experiments 1 to 9 was about 0.4 µg/ml. Since the highest concentrations of LH in blood of cycling ewes has been recorded at oestrus as about 100 ng/ml ( Geschwind & Dewey, 1969 ), it can be seen that artificially high doses of LH were used to provoke the responses seen during LH stimulation.

Both blood flow and secretion rate rose significantly while LH was infused ( see figures 17 and 18 ). However, the responses were variable. A number of factors could have contributed to this result: unequal absorption of LH; the experiments were at different times of the breeding season; the degree of stress at cannulation could have varied; different endogenous levels of LH may have altered luteal sensitivity; the corpora lutea had been maintained for various times since their formation; and finally, previous experiments and infusions may have affected the results of later infusions. It was not possible to correlate the percentage increases or the types of responses with season, the mean secretion rate, or with the pattern of blood flow.

Blood flow from the preparation is derived from the skin loop as well as the ovary ( chapter 2 ). The increases in blood flow observed may therefore not reflect an action of LH on the ovary, but of unknown factors on the capillary blood flow of the skin loop. However, LH does increase ovarian blood flow in the rat ( Wurtman, 1964 ), and in the ewe at operation ( Hixon & Clegg, 1969; Cook, Kaltenbach, Niswender, Norton & Nalbandov, 1969 ). The pattern of the blood flow response is different from that of the secretion rate response, and it is suggested that the two effects are mediated separately ( McCracken, Uno, Goding, Ichikawa & Baird, 1969 ).

The secretion rate response to LH shows a short term peak ( see figure 18 ). A similar response was reported to be caused by a single injection ( Short, McDonald & Rowson, 1963 ). Others who have investigated sheep ovarian progesterone secretion failed to note this characteristic, presumably because blood samples were not taken as frequently as here. The nature of the response readily explains why others have found it so difficult to demonstrate a response to LH ( see especially Cook *et al.*, 1969 ). Similar peaks in secretion rate have been observed at the start of continuous infusions of ACTH into the adrenal *in vitro* ( Urquhart & Li, 1968 ), of HCG into the testis *in vivo* ( Connell & Eik-Nes, 1969 ), and of LH into the bovine ovary *in vitro* ( Bartosik & Romanoff, 1969 ).

The short-lived response may be caused by the high doses of LH used, and it could be the result of several mechanisms. The burst of progesterone secretion may represent release of stored progesterone. However, this explanation seems unlikely in

view of the short turnover time of ovarian progesterone ( Short, McDonald & Rowson, 1963 ). In experiments 1 to 9, the extra secretion of progesterone averages 50 µg. This amount is well in excess of the content of one corpus luteum ( Stormshak, Inskeep, Lynn, Pope & Casida, 1963; Deane, Hay, Moore, Rowson & Short, 1966 ). It may be inferred that increased secretion is the result of increased synthesis. An alternative explanation is that LH which is bound to luteal receptors ( Rajaniemi & Vanha-Perttula, 1972 ), is metabolized to inactive products while still masking the binding sites. Much more likely, luteal tissue secretes maximally under the influence of the low endogenous concentrations of LH. Any further increase may be limited by supplies of precursors. In vitro, it has been reported that luteal progesterone is mainly synthesized from stored cholesterol ( Armstrong, 1968 ). Depletion of ovarian cholesterol is a well known effect of LH in vivo, and depletion of precursor cholesterol could account for the decline in progesterone secretion rate seen towards the end of LH infusion and after the infusion has been stopped ( see figure 18c ).

The half-life of progesterone in the sheep circulation is only a few minutes ( Short & Rowell, 1962 ), consequently, the short term peak in progesterone secretion can be of no physiological significance. During LH infusion, progesterone secretion fell after the peak, whereas the adrenal maintains a constant rate of cortisol secretion ofter a short term 'overshoot' ( Urquhart & Li, 1968 ). These differences in the pattern of secretion can be explained by the involvement of adrenal cortisol in feed-back inhibition of pituitary ACTH output. The pattern observed in figure 18c is consistent with

the idea that progesterone is not involved in a feed-back loop regulating LH during dioestrus. It is consistent with the idea that the sheep corpus luteum secretes autonomously ( Short, 1964 ).

Further studies on the action of LH on the sheep ovary are reported in chapters 4 and 6. In the light of the results obtained in these and other investigations, the role of LH as a luteotrophin in the ewe is discussed in chapter 8.

CHAPTER FOUR

# THE EFFECTS OF HUMAN CHORIONIC GONADOTROPHIN, PROLACTIN AND REPEATED INFUSIONS OF GONADOTROPHIN

## Introduction

In the preceding chapter, it was shown that the ovarian secretion response to continuous LH infusion was transitory. The short half-life of LH in the circulation, or a depletion of progesterone precursors could account for these findings.

The ability of the ovary to respond to a second infusion of gonadotrophin was tested, and the results of those experiments are described in this chapter.

The half-life of human chorionic gonadotrophin ( HCG ) is longer than that of LH in at least two species ( Parlow, 1968; Yen, Llerena, Little & Pearson, 1968 ). Moreover, HCG has many of the luteotrophic properties of LH ( see Evans & Simpson, 1950 ). The effects of HCG and LH infusion were therefore compared in order that the contribution made by the short half-life of LH to the transitory response, might be investigated.

Table 8.

Summary of experiments involving multiple infusions of gonadotrophins

| Expt. No. | Ewe No. | Date | Hormones infused first | Hormones infused second | Previous experiments | Comments |
|---|---|---|---|---|---|---|
| 10 | D342 | 29-10-70 | LH | LH | Expt. 6 on 10-9-70 1 mg LH, see chapter 3. | See chapter 3, expt. 8. |
| 11 | D314 | 27-9-70 | LH | LH | none | - |
| 12 | D342 | 7-1-71 | LH | LH | Expt. 15 on 12-11-70, see below. | - |
| 13 | D304 | 1-10-70 | HCG | LH | 1 mg LH on 24-6-70 | - |
| 14 | D305 | 24-9-70 | LH | HCG | PMSG & HCG treatment May 1970 | See chapter 3, expt. 7. |
| 15 | D342 | 2-11-70 | LH | prolactin | Expt. 10 on 29-10-70, see above. | See chapter 3, expt. 9. |
| 16 | D342 | 11-3-71 | LH | prolactin + LH | Expt. 12 on 7-1-71, see above. | - |

It is known that LH depletes ovarian cholesterol stores ( see Armstrong, 1968 ). However, prolactin has been shown to reverse this effect of LH and to increase the sensitivity of rabbit ovarian tissue to LH ( Hilliard, Spies & Sawyer, 1969 ). If the transitory response is caused by a lack of stored cholesterol, then it might be possible to increase both the amount of precursor and luteal sensitivity by infusing prolactin.

Materials and methods

Four sheep bearing autotransplanted ovaries were used for the experiments described in this chapter. The dates of the experiments, and the previous hormone treatments of the ewes are listed in table 8.

Intra-arterial infusions, and collections of ovarian vein blood were carried out as described in chapter 2 by Dr. D.T. Baird. Progesterone was assayed as described in chapter 2.

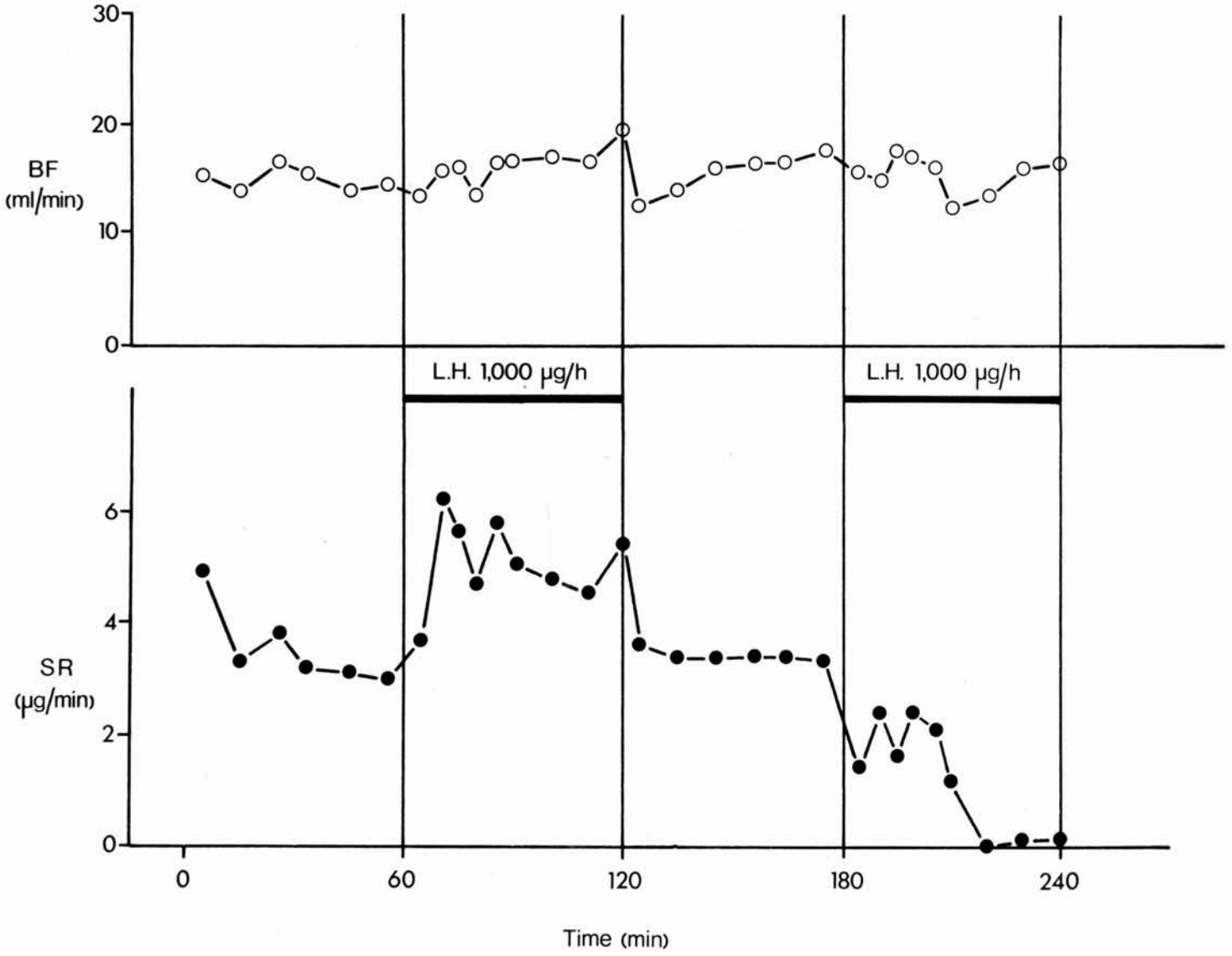
Infusion solutions were made on the day of use by dissolving the hormone preparations in physiological saline. They were sterilized as described in chapter 3. Solutions were infused at the rate of 17.3 ml/h.

Ovine luteinizing hormone was obtained from the National Institute of Health ( U.S.A. ). NIH-LH-S14 was used and its properties are listed in chapter 3.

Ovine prolactin was also a gift from the National Institute of Health. The batch used, NIH-P-S9, is stated by the institute to contain 30.3 I.U./mg as measured by the pigeon crop-sac weight method. Contamination with other hormones is stated to be low: FSH $<$0.018 NIH-FSH-S1 units/mg; LH $<$ 0.00036 NIH-LH-S1 units/mg; TSH $<$0.001 U.S.P. units/mg; and GH $<$0.01 U.S.P. units/mg.

Human chorionic gonadotrophin ( HCG ) was obtained as a sterile solution ( 1500 I.U./ml ) in water ('Pregnyl'; Organon Laboratories, Morden, Surrey ). 'Pregnyl' is made from human pregnancy urine. It was diluted with sterile physiological saline to give a solution of 200 I.U. in 17.3 ml.

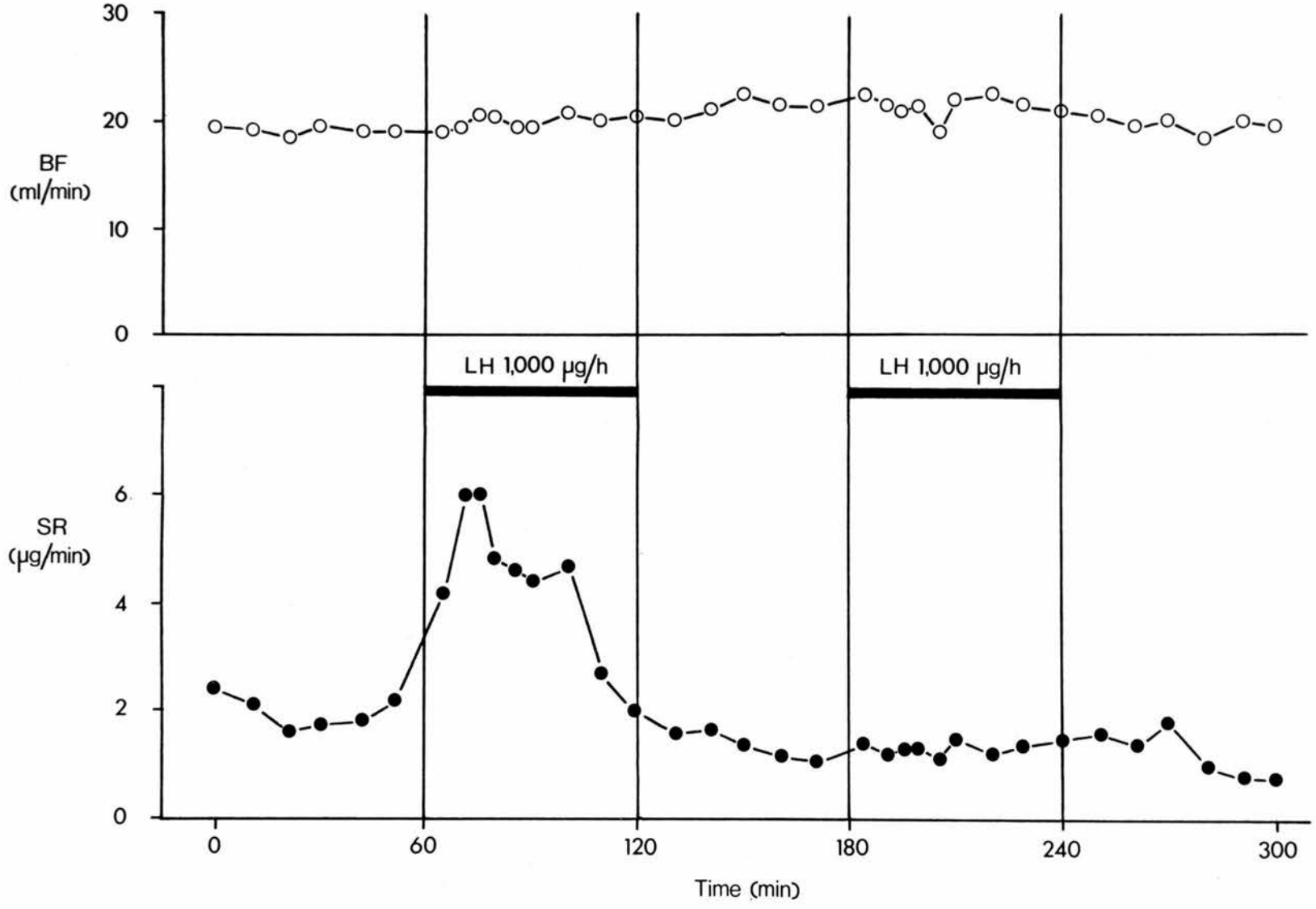
The design of each experiment can be seen from figures 19 to 25. Each one started with a control period lasting one hour. Saline was infused at the rate of 17.3 ml/h and 6 ovarian vein samples were collected at roughly ten-minute intervals of time. In the second hour of each experiment, gonadotrophin was infused, and 9 samples were collected at five-minute intervals during the first half hour, and then at ten-minute intervals during the second. The third hour was a control period in experiments 11 to 14, and 6 samples were taken. In these four experiments, the fourth hour was a period of gonadotrophin infusion, and 9 samples were collected in the same way as during the first gonadotrophin infusion. Samples were collected during a final control period in experiments 11, 12 and 13. In experiments 15 and 16, prolactin was infused for two hours, and samples were collected every 15 minutes after the first period of LH infusion. In the fifth hour of these two experiments, LH and prolactin were


30
20
10
0
BF
(ml/min)
L.H. 1,000 µg/h
L.H. 1,000 µg/h
6
4
2
0
SR
(µg/min)
0
60
120
180
240
Time (min)

Legend to figure 19

Responses to repeated luteinizing hormone infusions in experiment 10

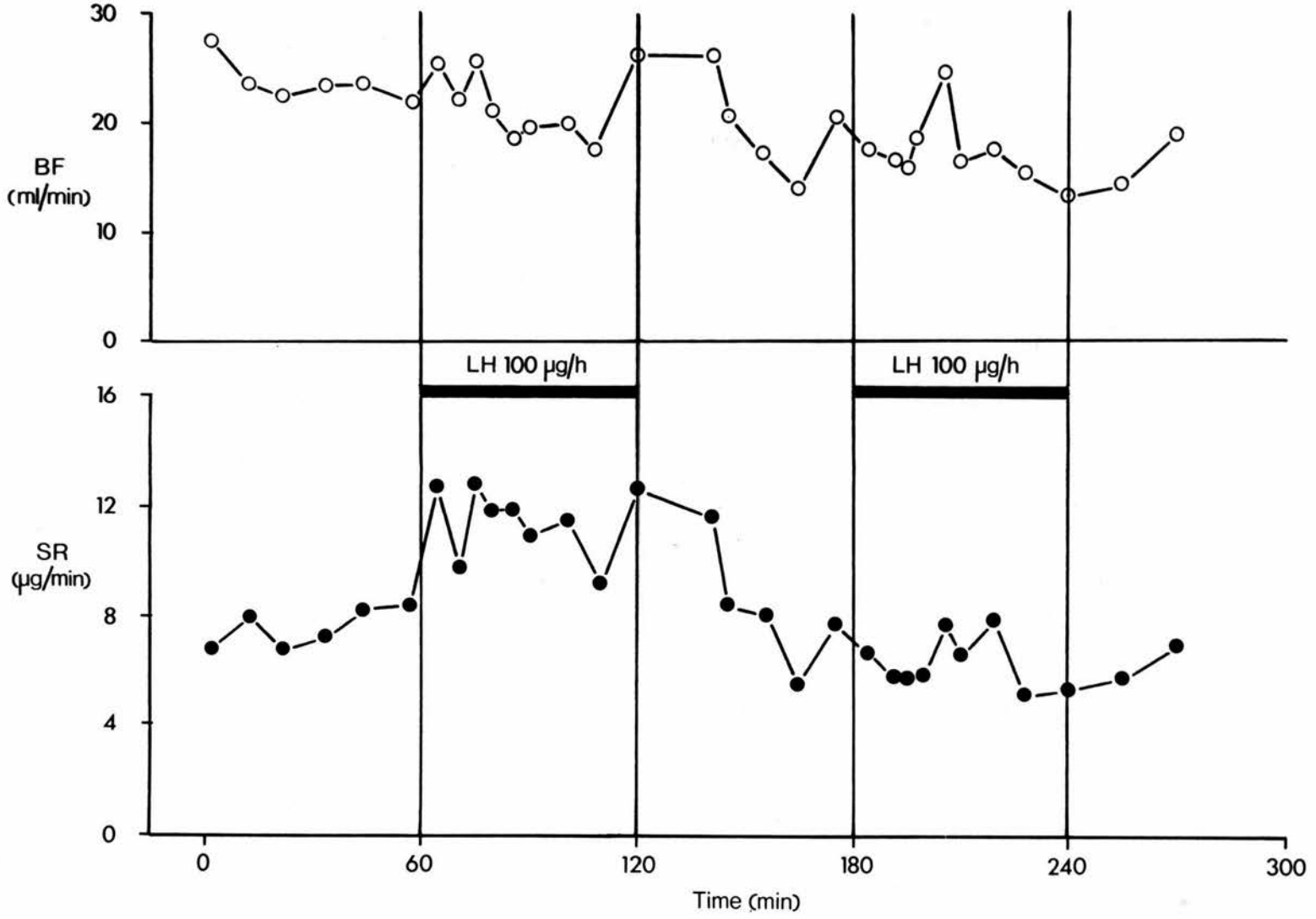
Changes in blood flow (BF) and in progesterone secretion rate (SR) in experiment 10. Horizontal bars indicate periods of gonadotrophin infusion.


30
20
10
0
BF
(ml/min)
LH 1,000 µg/h
LH 1,000 µg/h
6
4
2
0
SR
(µg/min)
0
60
120
180
240
300
Time (min)

Legend to figure 20

Responses to repeated luteinizing hormone infusions in experiment 11

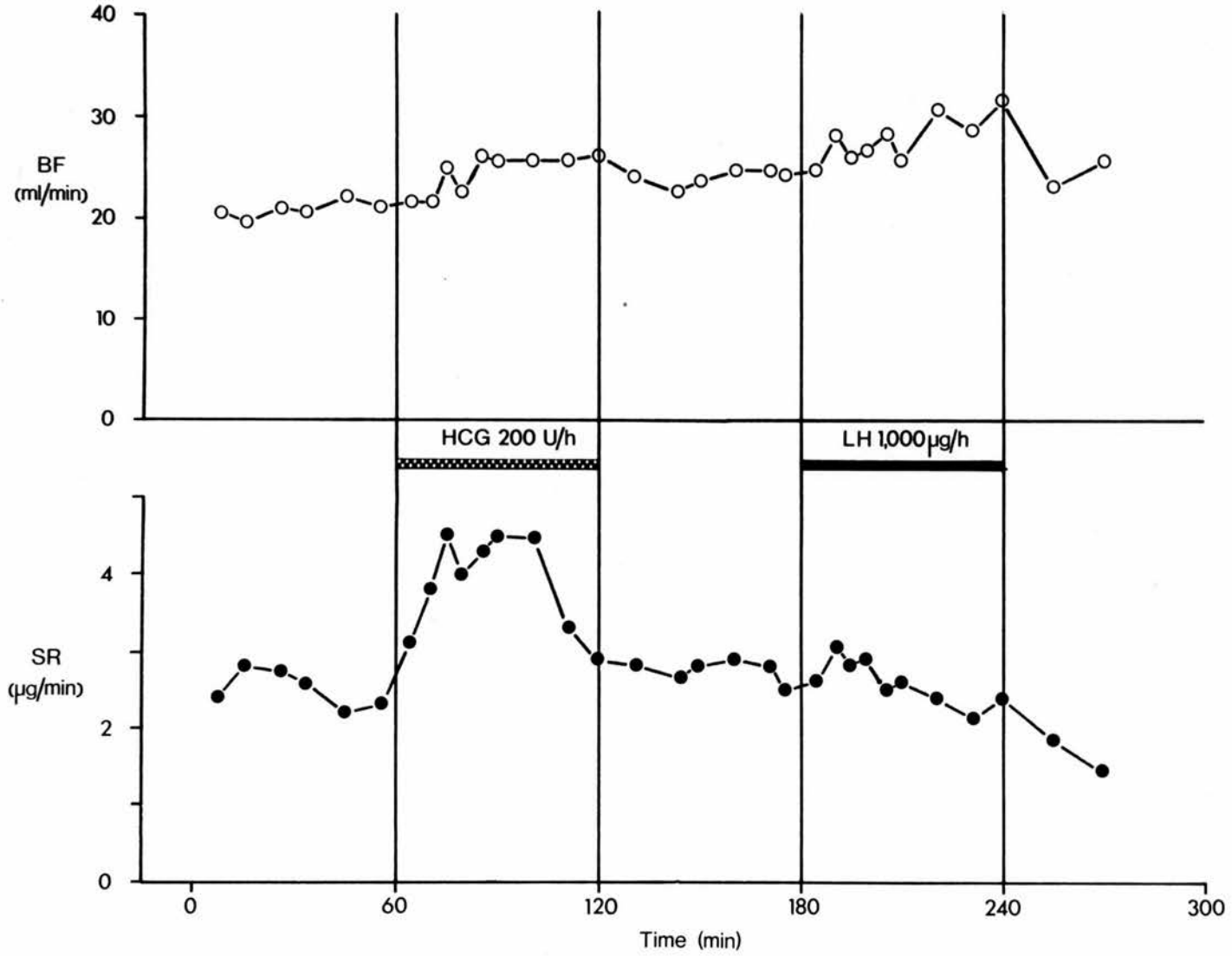
Changes in blood flow (BF) and progesterone secretion rate (SR) in experiment 11. Horizontal bars indicate periods of gonadotrophin infusion.


30
20
BF
(ml/min)
10
0
LH 100 µg/h
LH 100 µg/h
16
12
SR
(µg/min)
8
4
0
0
60
120
180
240
300
Time (min)

Legend to figure 21

Responses to repeated luteinizing hormone infusions in experiment 12

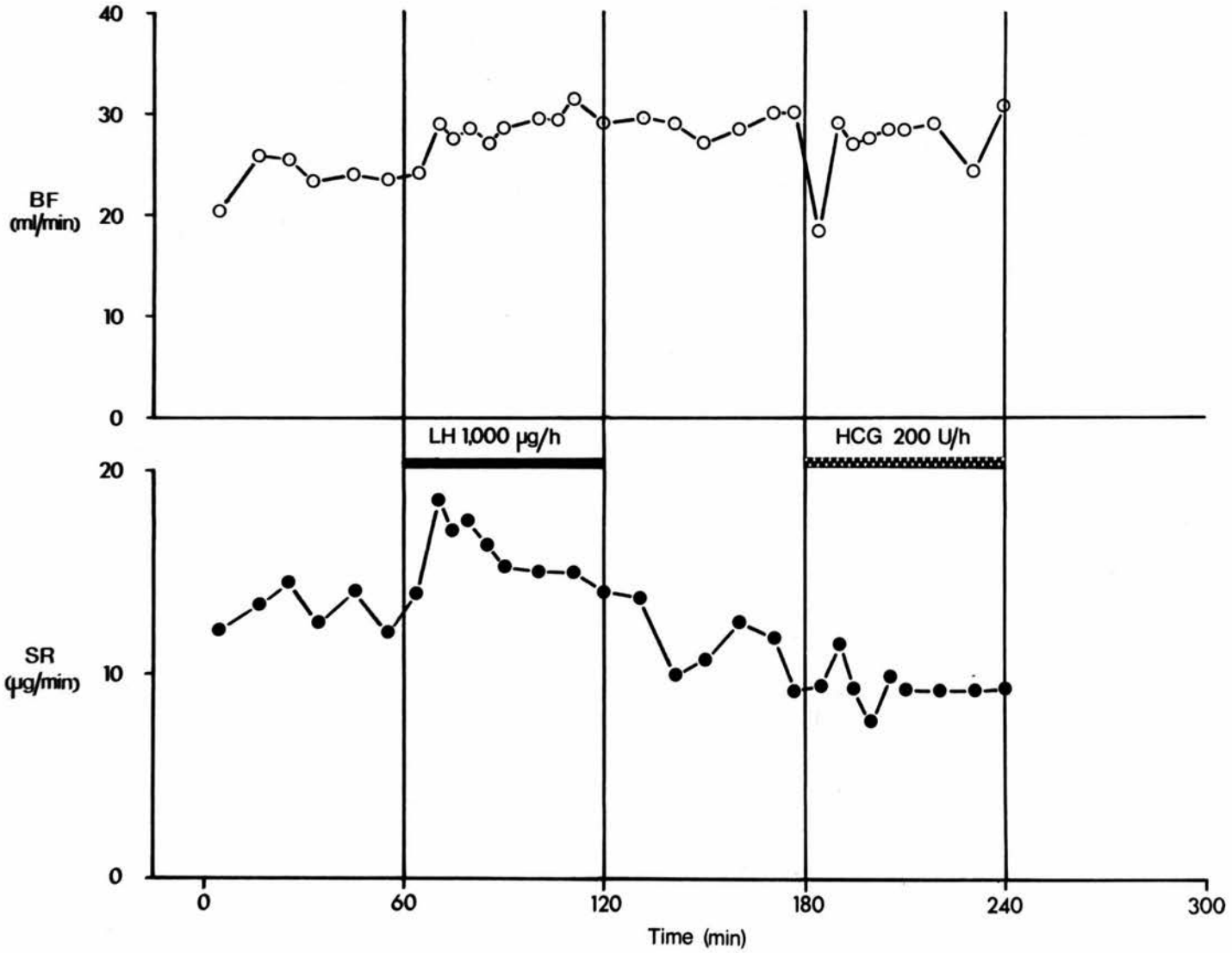
Changes in blood flow (BF) and progesterone secretion rate (SR) in experiment 12. Horizontal bars indicate periods of gonadotrophin infusion.


40
30
20
10
0
BF
(ml/min)
HCG 200 U/h
LH 1,000 µg/h
4
2
0
SR
(µg/min)
0
60
120
180
240
300
Time (min)

Legend to figure 22

Responses to human chorionic gonadotrophin and luteinizing hormone infusions in experiment 13

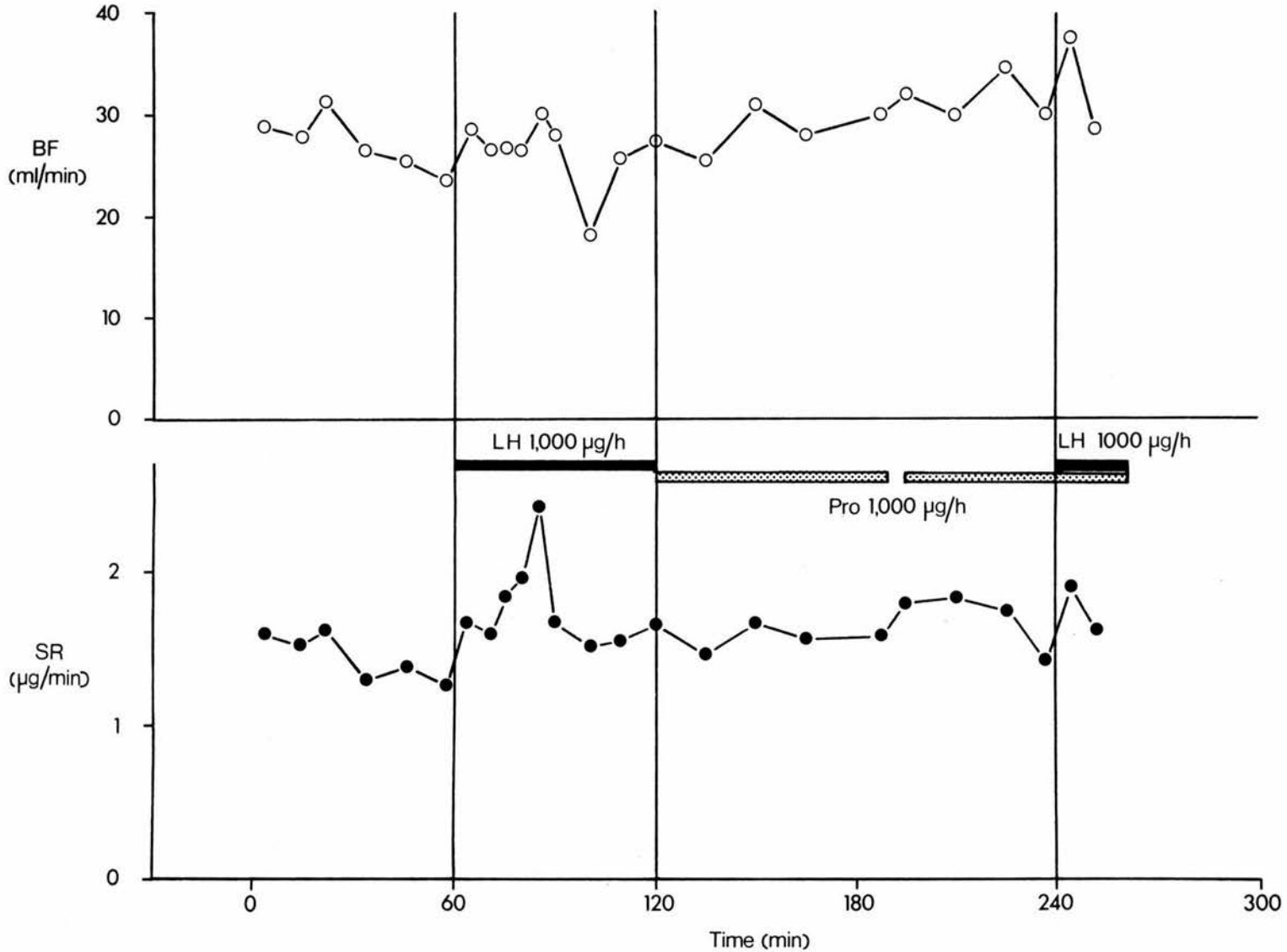
Changes in blood flow (BF) and progesterone secretion rate (SR) in experiment 13. Horizontal bars indicate periods of gonadotrophin infusion.


40
30
20
10
0
BF
(ml/min)
LH 1,000 µg/h
HCG 200 U/h
20
10
0
SR
(µg/min)
0
60
120
180
240
300
Time (min)

Legend to figure 23

Responses to luteinizing hormone and human chorionic gonadotrophin infusions in experiment 14

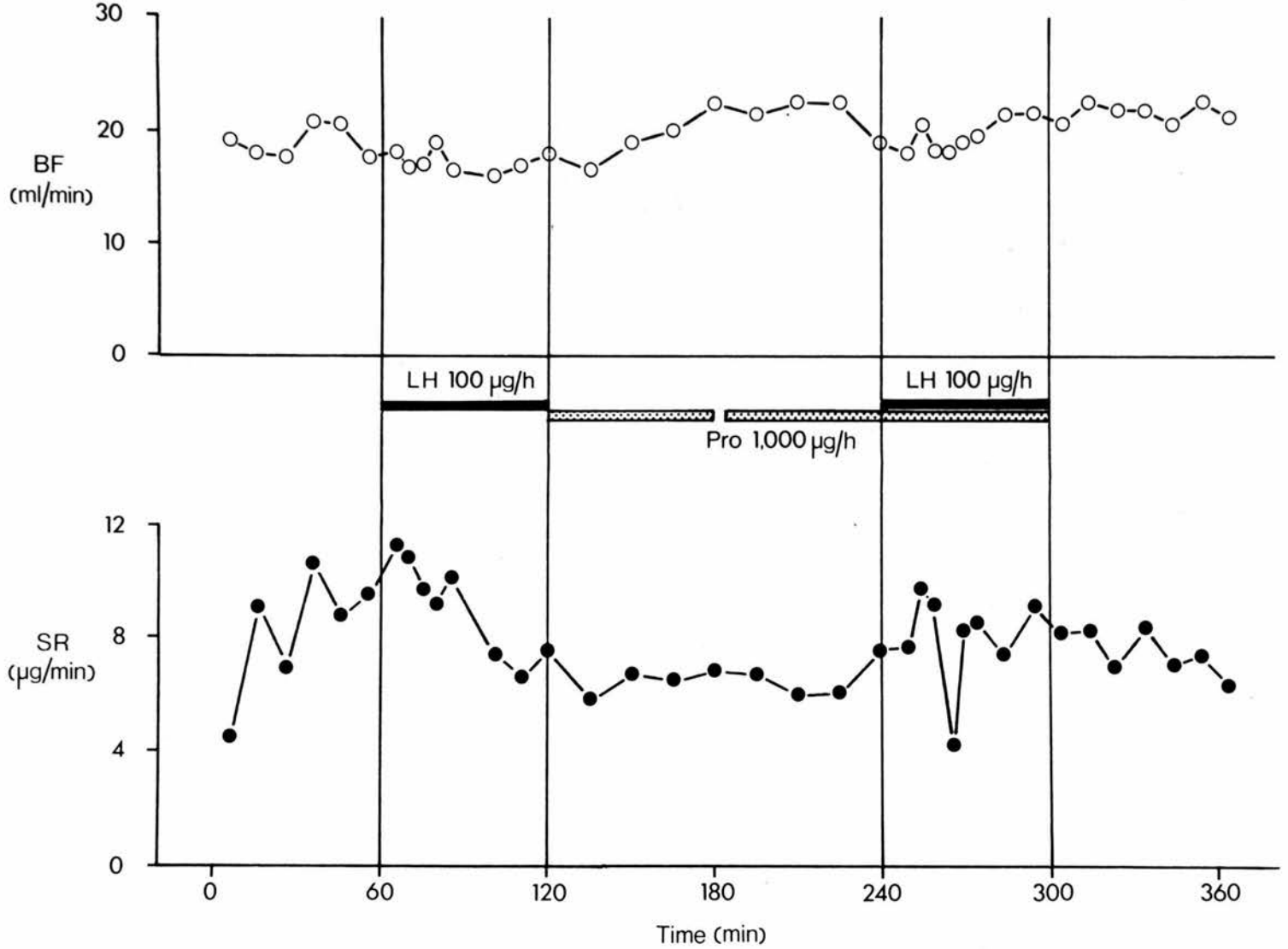
Changes in blood flow (BF) and progesterone secretion rate (SR) in experiment 14. Horizontal bars indicate periods of gonadotrophin infusion.


40
30
20
10
0
BF
(ml/min)
LH 1,000 µg/h
LH 1000 µg/h
Pro 1,000 µg/h
2
1
0
SR
(µg/min)
0
60
120
180
240
300
Time (min)

Legend to figure 24

Responses to luteinizing hormone and prolactin infusions in experiment 15

Changes in blood flow (BF) and progesterone secretion rate (SR) in experiment 15. Horizontal bars indicate periods of gonadotrophin infusion.


30
20
10
0
BF
(ml/min)
LH 100 µg/h
LH 100 µg/h
Pro 1,000 µg/h
12
8
4
0
SR
(µg/min)
0
60
120
180
240
300
360
Time (min)

Legend to figure 25

Responses to luteinizing hormone and prolactin infusions in experiment 16

Changes in blood flow (BF) and progesterone secretion rate (SR) in experiment 16. Horizontal bars indicate periods of gonadotrophin infusion.

infused together. Experiment 15 was prematurely ended by an accident; but in 16, samples were collected throughout the fifth hour, and during a final control period.

The dose rate chosen for HCG was intended to be one which would have the same effect as 1000 μg/h LH. In the rapid rat ovarian hyperaemia test, it was shown that 4.3 μg NIH-LH-S1 was as potent as 1 I.U. HCG ( Parlow, 1968 ). Since NIH-LH-S1 and NIH-LH-S14 are nearly equipotent, 200 I.U./h was chosen as the rate for infusion.

The first response to LH in three of these experiments ( 10, 14 and 15 ) was used in the characterization of the ovarian response reported in chapter 3.

Results

The changes in blood flow and secretion rate for each experiment are illustrated in figures 19 to 25.

In experiments 10 and 11, the first infusion of gonadotrophin significantly ( $P<0.025$ ) increased progesterone secretion. Blood flow also rose gradually. The pattern of the two responses was as described in chapter 3. The second infusion of LH failed to increase either blood flow or secretion rate. In 10, the secretion rate fell sharply, whereas in 11, levels were maintained.

In experiment 12, the blood flow was irregular, and values became lower towards the end of the experiment. The lower dose rate

of LH caused an increase in secretion rate during the first period of LH infusion, but not in the second.

Experiments 13 and 14 show that HCG has the same effect as LH. When HCG was infused first, it produced a transient rise in secretion rate, and blood flow rose progressively. Thereafter, the ovary was refractory to further stimulation with LH. LH was able to block a further response to HCG ( see figure 23 ).

In experiment 15, LH produced a short-lived secretion response. Prolactin infusion produced a slow rise in blood flow and secretion rates were steady. The experiment was terminated after an accident before any useful results could be obtained during the simultaneous infusion of LH and prolactin.

The control period secretion rate values in experiment 16 were particularly variable, and no significant increase in secretion rate was observed during the following period of LH infusion. Blood flow rose, and progesterone secretion remained steady during the two hours of prolactin infusion. There was an increased rate of secretion when LH and prolactin were infused together ( $P < 0.025$ ). Secretion rate fell slightly during the final control period.

Discussion

The transplanted ovaries tested in these experiments contained at least one corpus luteum of unknown age maintained because of the physical separation of the ovary from the uterus ( see

chapter 2 ). It is possible that the results obtained here might only apply to such maintained corpora lutea. The high secretion rates found in experiments 12, 14 and 16 are likely to have been produced from multiple corpora lutea.

Experiments 10, 11 and 12 clearly establish that the ovary becomes refractory to further stimulation with LH. The abrupt decline in secretion rate seen in experiment 11 may have been caused by the infusion of a small air bubble. In chapter 3, it was pointed out that the dose rates of LH being used were far in excess of those normally experienced by the ovary. Thus the refractory period may be a response found only when very high doses of LH are given. A similar period of ovarian refractoriness has been observed after LH administration to both rats ( see Armstrong, 1968 ) and rabbits ( Hilliard, Spies & Sawyer, 1969 ).

HCG has an effect in the ovary indistinguishable from that of LH ( see figures 22 and 23 ). The pattern of the response of blood flow and secretion rate was the same as that seen with LH in chapter 3. Like LH, HCG produces ovarian refractoriness so that later infusion of LH failed to increase secretion rate ( see figure 22 ). HCG was unable to stimulate the ovary after prior LH infusion ( see figure 23 ). In experiment 13, however, LH did provoke a further increase in blood flow ( see figure 22 ). This experiment indicates that changes in blood flow and secretion rate are not related. It is concluded from these experiments that LH and HCG are likely to act on the same sites when they increase luteal progesterone secretion.

On the basis of the different rates of metabolism of LH and HCG noted in other species, it is assumed that HCG has a longer half-life than LH in the ewe. If this is so, the transient secretion rate response elicited in experiment 13 indicates that this characteristic of the response is not caused by the short half-life of the stimulating hormone.

The results of the two experiments involving prolactin are inconclusive. Although there was no increase in progesterone secretion during the period of prolactin infusion, levels remained steady, whereas the typical pattern was for values to fall after LH stimulation ( see chapter 3 ).

The responses to LH and prolactin infusion seen in experiment 16 may have occurred because the ovary failed to respond to the first LH infusion, or because the refractory period induced by 100 μg/h LH was less than two hours in duration.

In both prolactin experiments the concentration of prolactin in the blood perfusing the ovary was about 1 μg/ml. The highest concentrations of this hormone in peripheral blood of the cycling ewes are likely to be below 0.2 μg/ml ( Cumming, Brown, Goding, Bryant & Greenwood, 1972 ). Thus the concentrations of prolactin infused are well in excess of physiological concentrations of this hormone.

CHAPTER FIVE

# THE EFFECT OF PROSTAGLANDIN $F_{2\alpha}$

## Introduction

Although prostaglandin $F_{2\alpha}$ increases steroidogenesis in minces of rat ovaries *in vitro* ( Pharriss, Wyngarden & Gutknecht, 1968 ), the action of this substance *in vivo* is quite the reverse ( Pharriss & Wyngarden, 1969 ). In a large domestic species - the cow - *in vitro* experiments show that prostaglandin $F_{2\alpha}$ stimulates progesterone synthesis from luteal slices ( Speroff & Ramwell, 1970 ) whereas *in vivo* infusions of prostaglandin $F_{2\alpha}$ cause luteal regression ( Rowson, Tervit & Brand, 1972 ).

Infusion of prostaglandin $F_{2\alpha}$ directly into the autotransplanted ovary of the ewe leads to luteal regression ( McCracken, Glew & Scaramuzzi, 1970; Barrett, de Blockey, Brown, Cumming, Goding, Mole & Obst, 1971 ).

Four experiments involving prostaglandin $F_{2\alpha}$ were performed. It was hoped to confirm previous work on the ewe, and to show that prostaglandin $F_{2\alpha}$ infusion would regress old *corpora lutea* and allow the formation of a new one after ovulation had taken place on a known date.

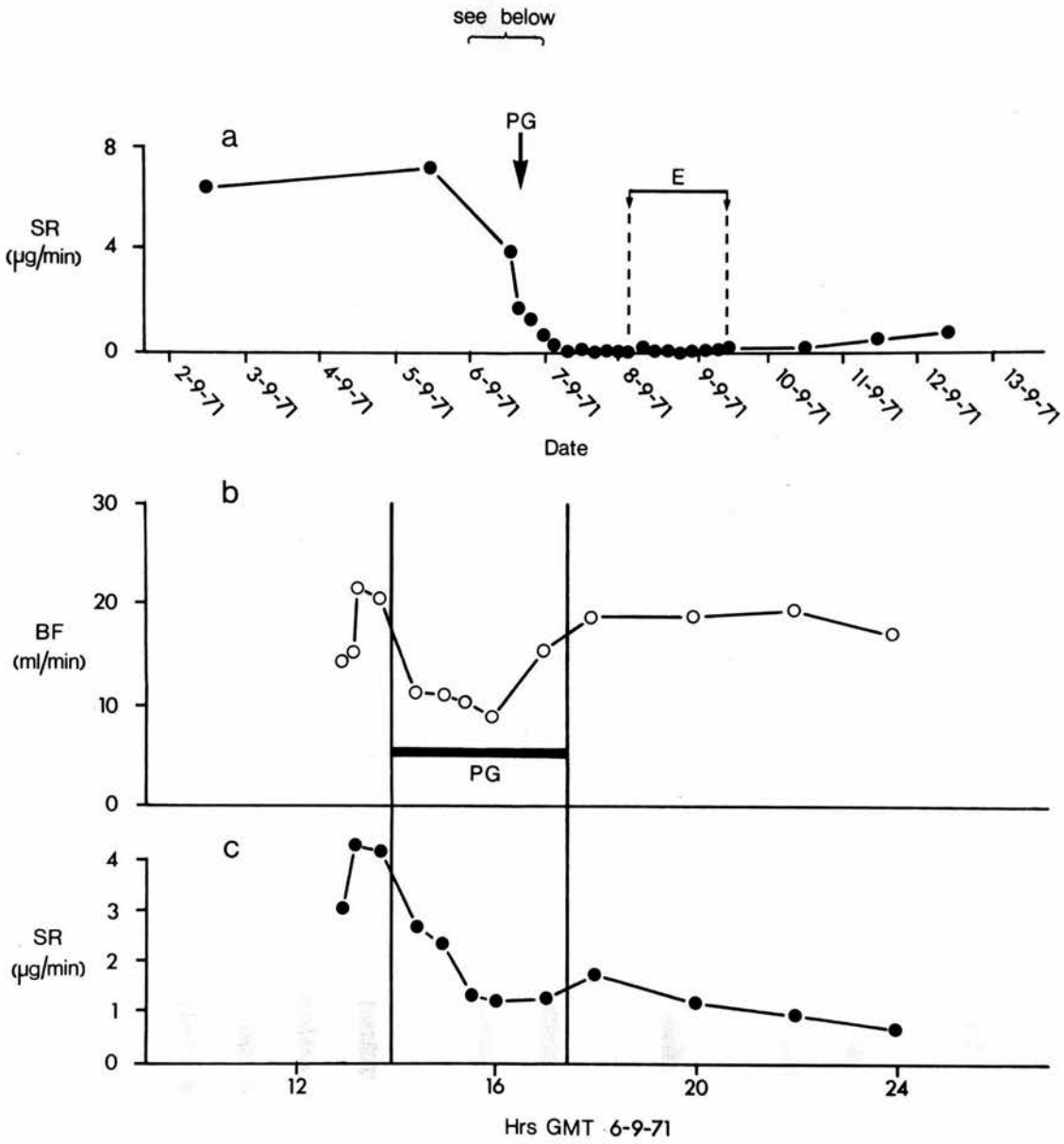
Materials and methods

Prostaglandin $F_{2\alpha}$ was supplied by Upjohn ( Kalamazoo, Michigan, U.S.A. ) as an ethanolic solution 1 mg/ml. On the day of use 0.35 ml was dried and dissolved in 67.2 ml sterile saline. Infusions of that solution were maintained for 3½ hours at a rate of 0.14 ml/min ( 44 µg/h ).

Four ewes bearing ovarian autotransplanted ovaries were prepared for intra-arterial infusion and for collection of ovarian vein blood as described in chapter 2 by Dr. D.T. Baird. Two of the ewes were treated with prostaglandin late in May 1971, and two late August 1971.

Ovarian vein samples were collected for four days after and one day before prostaglandin infusion. On the day of infusion, samples were collected at two-hourly intervals, or more frequently. On the following three days, the ewes were tested for heat and bled at four-hourly intervals. I am greatly indebted to Dr. R.B. Land and Mr. A.G. Wheeler for helping with collections of blood and testing for oestrus. Miss M. Fordyce also provided welcome technical assistance.

Progesterone concentrations in ovarian vein plasma samples were estimated by the CPB method outlined in table 1.


see below
a
PG
E
8
4
0
SR
(µg/min)
2-9-71
3-9-71
4-9-71
5-9-71
6-9-71
7-9-71
8-9-71
9-9-71
10-9-71
11-9-71
12-9-71
13-9-71
Date
b
30
20
10
0
BF
(ml/min)
PG
c
4
3
2
1
0
SR
(µg/min)
12
16
20
24
Hrs GMT 6-9-71

Legend to figure 26

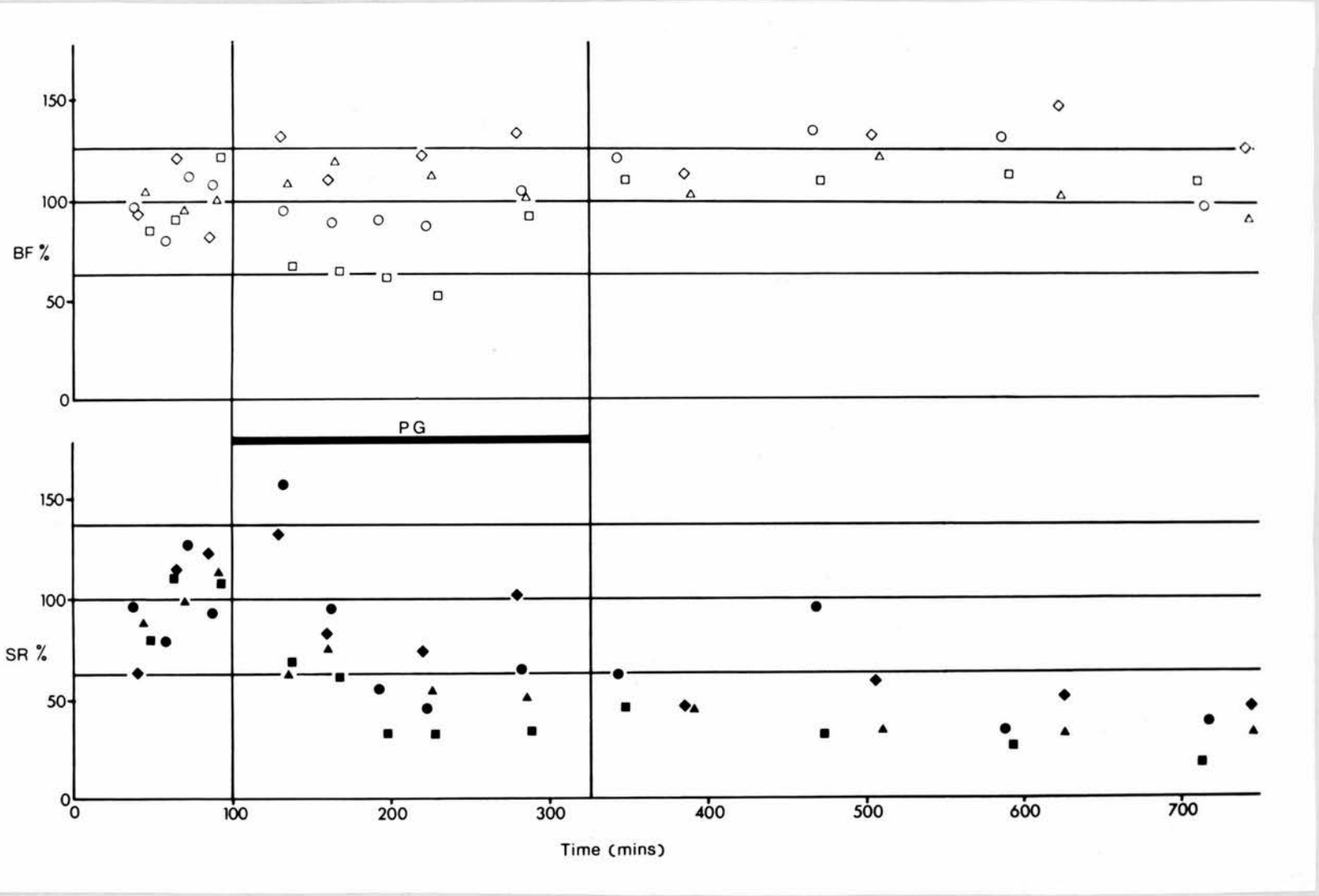
Effects of prostaglandin $F_{2\alpha}$ infusion

a), changes in progesterone secretion rate before and after the day of prostaglandin infusion.

b), changes in blood flow ( BF ) on the day of prostaglandin infusion.

c), changes in progesterone secretion rate ( SR ) on the day of prostaglandin infusion.

Experiment G was conducted on MX9 during September 1971. In (a), 'PG' indicates the time of prostaglandin infusion, and 'E' the duration of oestrus. In (b) and (c), the horizontal bar shows the duration of prostaglandin ( PG ) infusion at the rate of 44 μg/h.


150
100
BF %
50
0
PG
150
100
SR %
50
0
0
100
200
300
400
500
600
700
Time (mins)

Legend to figure 26A

Effects of prostaglandin $F_{2\alpha}$ infusion

Mean relative changes in blood flow ( BF ) and progesterone secretion rate ( SR ) in four experiments involving prostaglandin $F_{2\alpha}$ infusion at the rate of 44 µg/h.

△ ▲ results from MX9 on 31-5-71.

□ ■ results from MX9 on 6-9-71.

◇ ◆ results from D304 on 1-6-71.

○ ● results from D304 on 6-9-71.

Results

The results of one of the experiments are illustrated in figure 26. In all four experiments, the changes in progesterone secretion during the day of the infusion were very similar to those shown in the lower part of figure 26. The changes in blood flow observed during infusion of prostaglandin were variable, and the fall shown in figure 26 was not typical.

The longer term changes in progesterone secretion rate are shown in the upper half of figure 26. Both ewes treated in late August showed this pattern. Neither of the ewes treated late in May showed evidence for the formation of a new corpus luteum, because the progesterone secretion rate remained very low after the infusion.

Neither ewe treated in late May returned to oestrus, presumably because this date is outside the normal breeding season. One of the ewes treated during late summer showed heat, and the other showed signs of pro-oestrus but did not allow the ram to mate her.

Discussion

The results fully confirm two previous reports ( McCracken, Glew & Scaramuzzi, 1970; Barrett, de Blockey, Brown, Cumming, Goding, Mole & Obst, 1971 ).

The occurrence of oestrus, and a subsequent rise in progesterone secretion are indicative of ovulation and the formation of a

new corpus luteum. The fact that the two ewes treated in May were outside their normal breeding season probably accounts for their failure to return to oestrus. The flock from which the Merino ewes were taken have a high proportion of 'silent' heats ( A.G. Wheeler, unpublished ), and this would explain why only one of the ewes treated late in the summer would allow the ram to mate her.

Pharriss ( 1970 ) proposed that the luteolytic effect of prostaglandin $F_{2\alpha}$ was caused by a selective venoconstriction of the vessels draining the corpus luteum. The results obtained for blood flows are in conflict with that suggestion, unless prostaglandin $F_{2\alpha}$ also causes a redistribution of blood between the ovary and the skin, or between luteal and non-luteal compartments of the ovary.

Infusion of prostaglandin $F_{2\alpha}$ directly into the autotransplanted ovary leads to a series of changes in the levels of at least three hormones ( Barrett, de Blockey, Brown, Cumming, Goding, Mole & Obst, 1971 ). Those changes are very similar to those occurring around oestrus in spontaneously cycling utero-ovarian transplants ( McCracken, Glew & Levy, 1970 ), in ewes where the utero-ovarian vein has been anastomosed to the mammary vein ( Cox, Mattner & Thorburn, 1971 ), and in normal ewes ( Moore, Barrett, Brown, Schindler, Smith & Smyth, 1969; Scaramuzzi, Caldwell & Moor, 1970; Bjersing, Hay, Kann, Moor, Naftolin, Scaramuzzi, Short & Younglai, 1972 ).

A recent review concluded that the action of prostaglandin $F_{2\alpha}$ in vivo in a number of species was on the corpus luteum alone ( Pharriss, Tillson & Erickson, 1972 ). In the cow, the fertility of animals mated at oestrus after prostaglandin $F_{2\alpha}$ induced regression was not lower than that of cows mated at spontaneous oestrus ( Rowson, Tervit & Brand, 1972 ).

Finally, a natural uterine luteolytic factor present in uterine vein blood of sheep has been identified as prostaglandin $F_{2\alpha}$ ( McCracken, Carlson, Glew, Goding, Baird, Gréen & Samuelsson, 1972 ). The above evidence suggests that non-luteal structures suffer no damage during prostaglandin $F_{2\alpha}$ infusion.

It is concluded that prostaglandin $F_{2\alpha}$ infusion causes complete luteal regression at 44 $\mu g$ / h. During the breeding season, removal of the old corpus luteum allows ovulation and the formation of a new corpus luteum on a defined date. Prostaglandin $F_{2\alpha}$ infusion was used in the experiments described in the next chapter.

CHAPTER SIX

# VARIATIONS IN LUTEAL SENSITIVITY

## Introduction

The ovarian responses reported in chapter 3 were variable. Factors which could have contributed to that result were considered. In this chapter, two of those variables are investigated. The work described in this section is an attempt to test whether corpora lutea become more responsive in terms of progesterone secretion as they become older, or after they have been previously treated with LH.

PMSG and HCG treated ewes had been studied in the early work on the transplanted ovary ( McCracken, Uno, Goding, Ichikawa & Baird, 1969 ). However, gonadotrophin treatment may have caused the ovaries to respond abnormally. It was considered an important possibility and further investigation might indicate the physiological significance of earlier results. As it was difficult to separate the effects of increasing age from those of prior LH treatment, it was decided to test both simultaneously. The project was started when a reliable means of causing luteal regression became available ( see chapter 5 ). Although oestrogen is luteotrophic in rabbits, there is much evidence that oestrogen may also be luteolytic particularly in

the ewe. In-vitro work on purified bacterial enzymes ( Talahay & Marcus, 1956 ), on porcine luteal tissue ( Cook, Niswender, Sutterlin, Norton & Nalbandov, 1968 ), on bovine tissue ( Masaracchia & Gawienowsky, 1969 ), and on ovine corpora lutea ( Akbar, Stormshak & Lee, 1972 ), has demonstrated that oestrogens may inhibit progesterone synthesis. In addition, the destruction of follicles allows smaller doses of LH to maintain corpora lutea in hypophysectomized ewes ( Karsch, Noveroske, Roche, Norton & Nalbandov, 1970 ). These workers suggested that the follicles and corpora lutea compete for a limiting amount of LH. However, work on the rat indicates that oestrogens can sensitize the ovary to the action of gonadotrophins ( Simpson, Evans, Fraenkel-Conrat & Li, 1941; Nikitovitch-Winer, 1965 ). Oestrogen secretion rates were determined in order to see if there were any correlation between luteal sensitivity and endogenous oestrogen production.

Materials and methods

Luteal responsiveness was tested under four conditions: 1, 11 or 12 days after prostaglandin infusion, 'young corpora lutea'; 2, 46 or 48 days after prostaglandin infusion and 35 or 36 days after LH infusion, 'maintained corpora lutea after prior LH infusion'; 3, 42 days after prostaglandin infusion,'maintained corpora lutea without prior LH'; 4, 70 days after prostaglandin infusion and 28 days after LH infusion, 'aged-maintained corpora lutea after LH infusion'. The dates of infusions and the animals used for these experiments are set out in table 9. The availability of shared facilities on the farm dictated that the intervals between infusions were not quite equal, but these minor differences are not important.

Table 9.

Dates of LH and prostaglandin (PG) infusions in experiments to test variations in luteal sensitivity

| Ewe No. | Date of PG infusion | First LH infusion | | | Second LH infusion | | |
|---|---|---|---|---|---|---|---|
| | | Expt. No. | Date | Days since PG | Expt. No. | Date | Days since PG |
| D304 | 6-9-71 | 17 | 17-9-71 | 11 | 18 | 22-10-71 | 46 |
| D305* | 16-3-72 | 19 | 28-3-72 | 12 | 20 | 3-5-72 | 48 |
| MX9* | 23-8-72 | 21 | 4-10-72 | 42 | 22 | 1-11-72 | 70 |
| D342* | 24-8-72 | 23 | 5-10-72 | 42 | 24 | 2-11-72 | 70 |

* These ewes were on heat two days after prostaglandin infusion.

Luteinizing hormone (LH) and prostaglandin $F_{2\alpha}$ (PG) were obtained as previously described. In some experiments, NIH-LH-S17 was used. This batch is stated to be 1.01 times as potent as the NIH-LH-S1 preparation. Contamination with other activities is the same or less than for NIH-LH-S14. D304 and D305 were perfused with NIH-LH-S14, and D342 and MX9 with NIH-LH-S17.

Maintained corpora lutea were regressed by intra-arterial PG infusion. The method was described in chapter 5. The ewes were tested for oestrus twice daily and peripheral blood samples were taken once a day. For setting up these infusions I am indebted to Dr. D.T. Baird, and for the blood sampling and testing for oestrus I wish to thank Dr. R.B. Land, Mr. A.G. Wheeler and Miss M. Fordyce. Each of the four ewes showed a fall in the concentration of progesterone in peripheral plasma samples after PG infusion. The changes were characteristic of ewes at oestrus in every case.

Luteal responsiveness was tested at different times after luteal formation by infusing LH at three dose rates for separate one hour periods. For these experiments, LH solutions were not sterilized, and as an added precaution, the solutions contained inert protein. LH was dissolved in saline containing 5 % v/v peripheral plasma freshly drawn from the experimental ewe. RIA of the infusion solutions confirmed that the correct dose of LH was delivered. During control periods, vehicle was infused at the rate of 11.3 ml/h. LH solutions were infused at the same rate to give dose rates of 10, 100 and 1000 µg/h. Each experiment was divided into six hour-long periods so that each LH infusion was preceded by a control period ( see figures 27 - 30 ).

Table 10.

Oestrogen secretion rates (SR) from two samples from the first control period of infusions to test variations in luteal sensitivity

| Expt. No. | State of *corpora lutea* | Prior LH ? | Oestrogen SR pg / min | | Details |
|---|---|---|---|---|---|
| 17 | young | no | 339 | < 277 | figure 27 |
| 19 | young | no | 346 | 240 | figure 27 |
| 18 | maintained | yes | 264 | 77 | figure 28 |
| 20 | maintained | yes | 242 | 239 | figure 28 |
| 21 | maintained | no | 1326 | 624 | figure 29 |
| 23 | maintained | no | 331 | 292 | figure 29 |
| 22 | aged-maintained | yes | 262 | <178 | figure 30 |
| 24 | aged-maintained | yes | 601 | 439 | figure 30 |

Cannulations of the jugular vein and carotid artery were performed by Dr. D.T. Baird or Dr. R.B. Land as described in chapter 2. Ovarian vein blood was collected as far as possible at ten-minute intervals by Dr. D.T. Baird, Dr. R.B. Land or the author throughout each experiment.

Ovarian vein plasma samples were assayed for progesterone as described in table 1, and peripheral plasma samples as described in table 2.

I am indebted to Dr. R.J. Scaramuzzi and Mrs. G. Heavon-Jones for oestradiol assays. Ovarian vein plasma samples ( 4 ml ) were assayed according to a previously described technique ( Abraham, Hopper, Tulchinsky, Swerdloff & Odell, 1971 ). An antiserum prepared by Dr. B.V. Caldwell was used. The method uses celite column chromatography, and it is specific for oestradiol-17β. As these assays took some time, it was decided to measure only two samples from each control period. The samples chosen for assay were selected by reference to a table of random numbers.

Calculations and statistical tests were carried out as described in chapter 2.

## Results

Table 10 shows the oestrogen secretion rates determined from analyses on two samples from the first control period of each experiment.

Table 11.

Mean progesterone secretion rate (PSR) and blood flow (BF) during the first control period of infusions to test variations in luteal sensitivity.

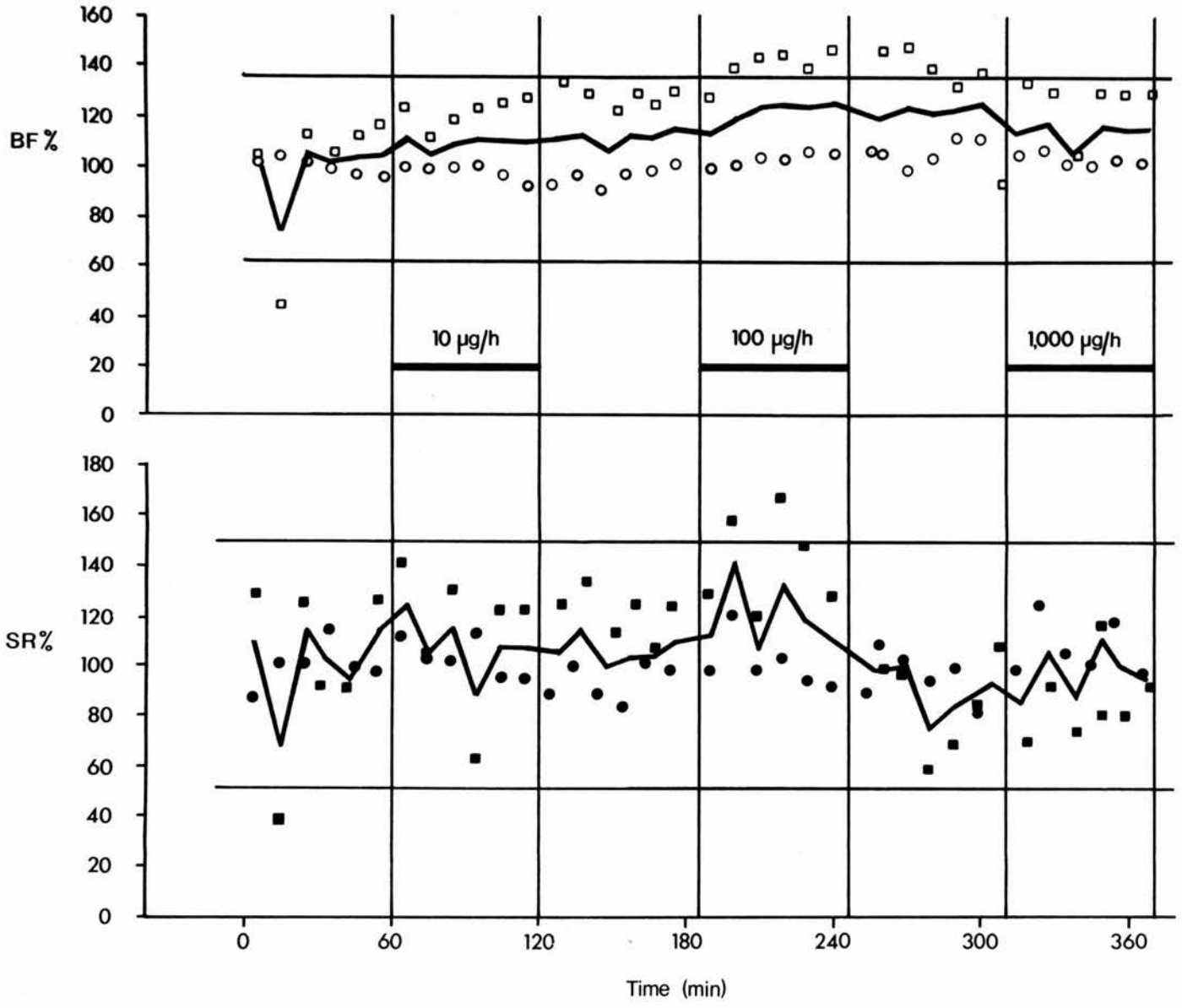
| Expt. No. | Ewe No. | BF* ml / min | PSR* µg / min |
|---|---|---|---|
| 17 | D304 | 24.9 ± 0.33 (6) | 2.0 ± 0.07 (6) |
| 18 | D304 | 18.9 ± 1.34 (6) | 2.1 ± 0.13 (6) |
| 19 | D305 | 13.4 ± 1.49 (6) | 2.0 ± 0.29 (6) |
| 20 | D305 | 12.3 ± 0.36 (6) | 4.6 ± 0.12 (6) |
| 21 | MX9 | 18.3 ± 0.19 (6) | 5.0 ± 0.27 (6) |
| 22 | MX9 | 21.5 ± 0.73 (6) | 3.0 ± 0.50 (6) |
| 23 | D342 | 23.8 ± 0.81 (7) | 4.1 ± 0.27 (7) |
| 24 | D342 | 25.0 ± 0.64 (6) | 8.3 ± 0.26 (6) |

*Mean ± standard error ( number of determinations ).

The changes in blood flow and progesterone secretion rate are illustrated in figures 27 to 30. The mean control levels of progesterone secretion rate and blood flow are set out in table 11.

There are no obvious differences in the patterns of change in blood flow between the four pairs of experiments. One ewe ( D305 ) failed to show a rise in blood flow during any period of LH infusion. In the remaining 6 experiments, blood flow rose during the infusion of 1000 μg/h LH in 5. There were three increases in response to 100 μg/h LH and only one response to 10 μg/h LH.

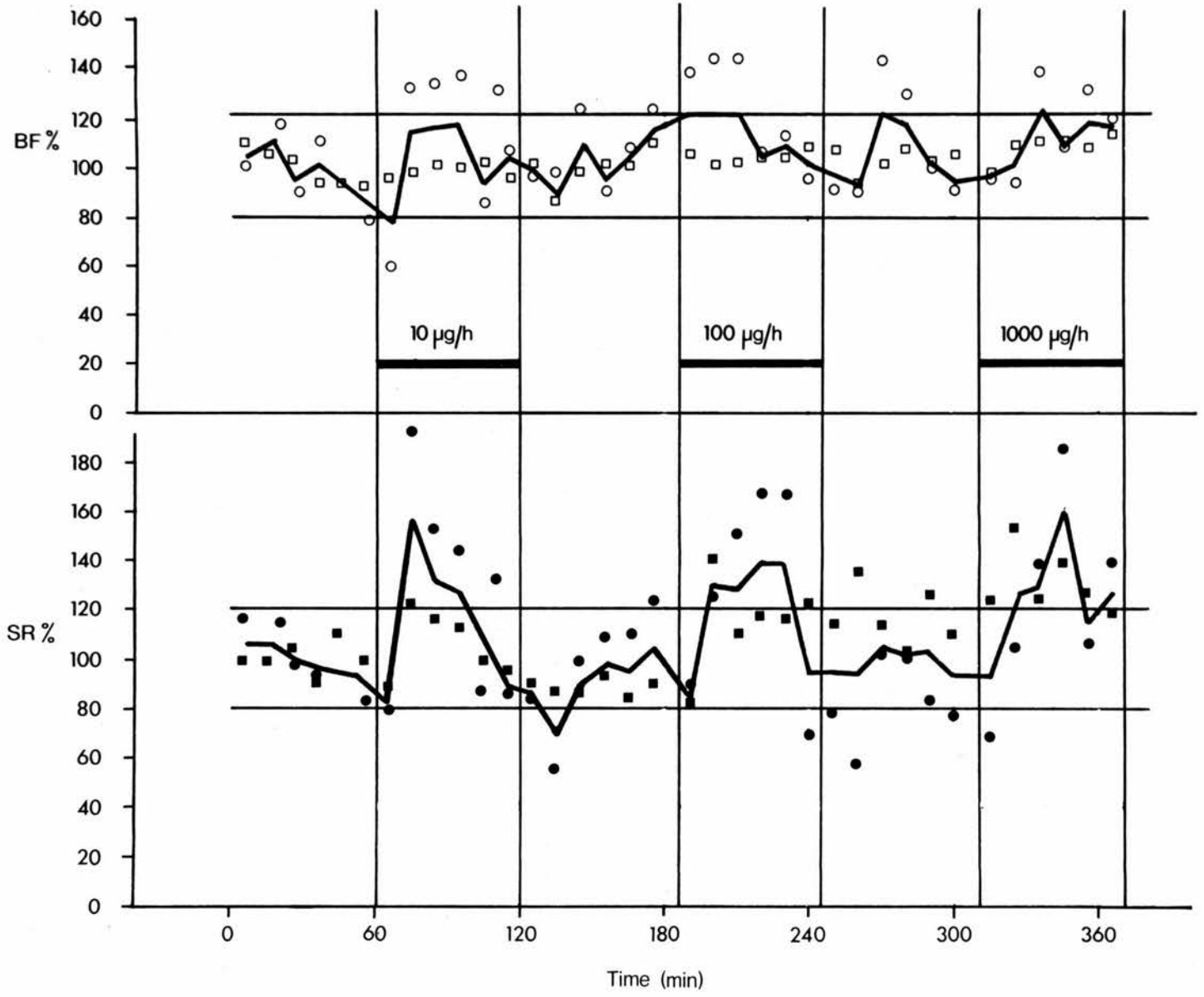
Figures 27 to 30 show that luteal responsiveness in terms of an increased progesterone secretion rate, varied with age and with prior LH treatment. Maintained corpora lutea previously treated with LH gave definite responses at all LH infusion rates ( see figure 28 ). In both individual experiments, a Student 't' test shows the mean progesterone secretion rate during periods of LH infusion to be higher ( $P < 0.01$ ) than that during control periods. The variability of the results in other experiments was disappointing, but it is clear that young corpora lutea are both less sensitive and less responsive ( see figure 27 ) than are corpora lutea under the other conditions tested. Maintained corpora lutea previously treated with LH appear to be more responsive than those of the same age that have not been previously infused with LH. In one of the pair of experiments on aged-maintained corpora lutea previously infused with LH, there was a rise in progesterone secretion rate during the infusion of 10 and 100 μg/h LH, whereas the other experiment gave a steady decline after an increase during the first LH infusion period ( see figure 30 ).


BF %
160
140
120
100
80
60
40
20
0
10 µg/h
100 µg/h
1,000 µg/h
SR%
180
160
140
120
100
80
60
40
20
0
0
60
120
180
240
300
360
Time (min)

Legend to figure 27

Responses of young corpora lutea to luteinizing hormone infusion

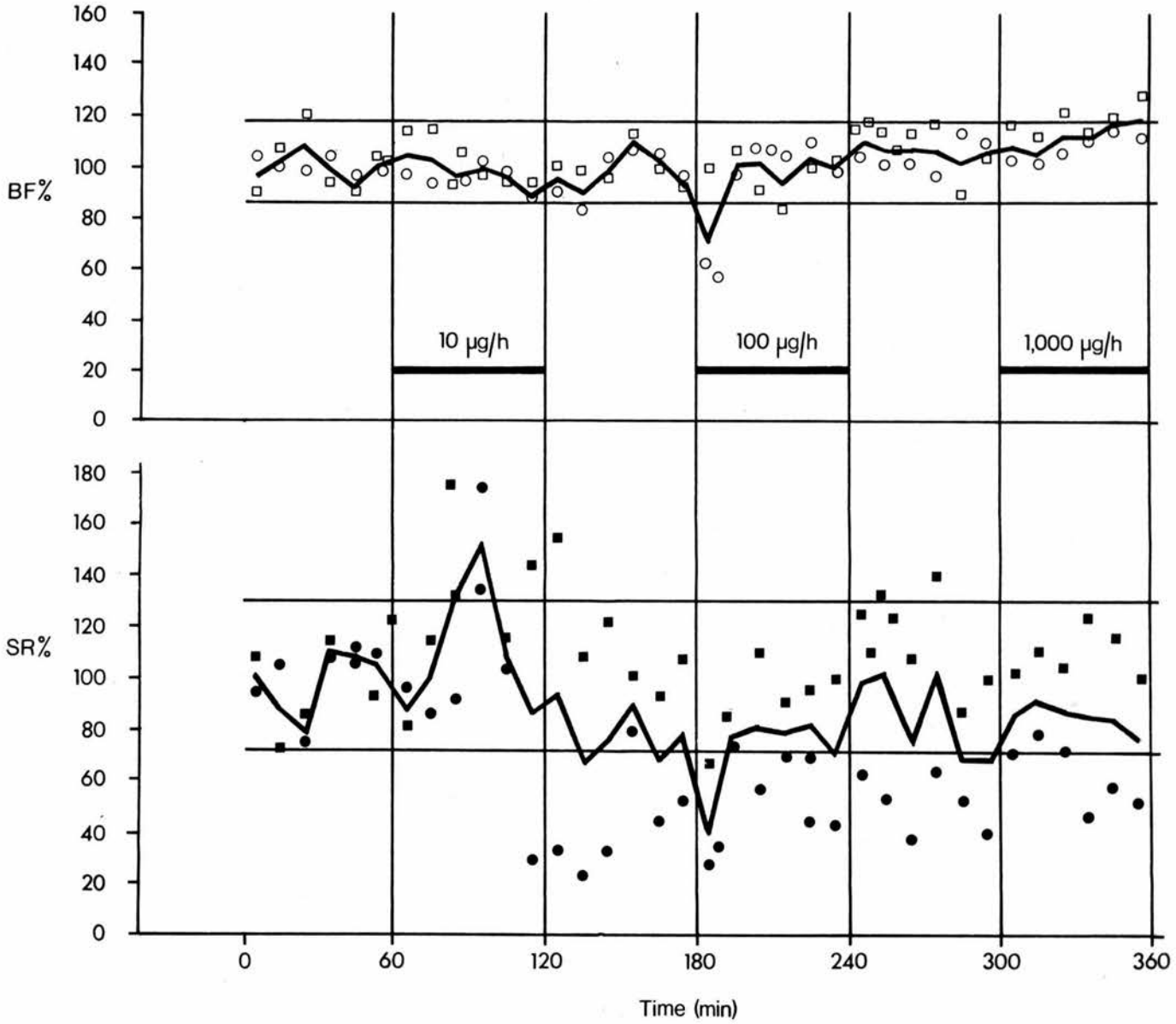
Mean relative changes in blood flow ( open symbols,) and in progesterone secretion rate ( solid symbols,) in two experiments ( experiment 17 circles, experiment 19 squares,) where young corpora lutea were tested for their responses to three rates of luteinizing hormone (LH) infusion. Horizontal bars indicate the duration of LH infusions. The horizontal lines indicate the 95 % control limits for values observed during the first hour of each experiment.


BF %
SR %
160
140
120
100
80
60
40
20
0
180
160
140
120
100
80
60
40
20
0
10 µg/h
100 µg/h
1000 µg/h
0
60
120
180
240
300
360
Time (min)

Legend to figure 28

Responses of maintained corpora lutea previously infused with luteinizing hormone to luteinizing hormone infusion

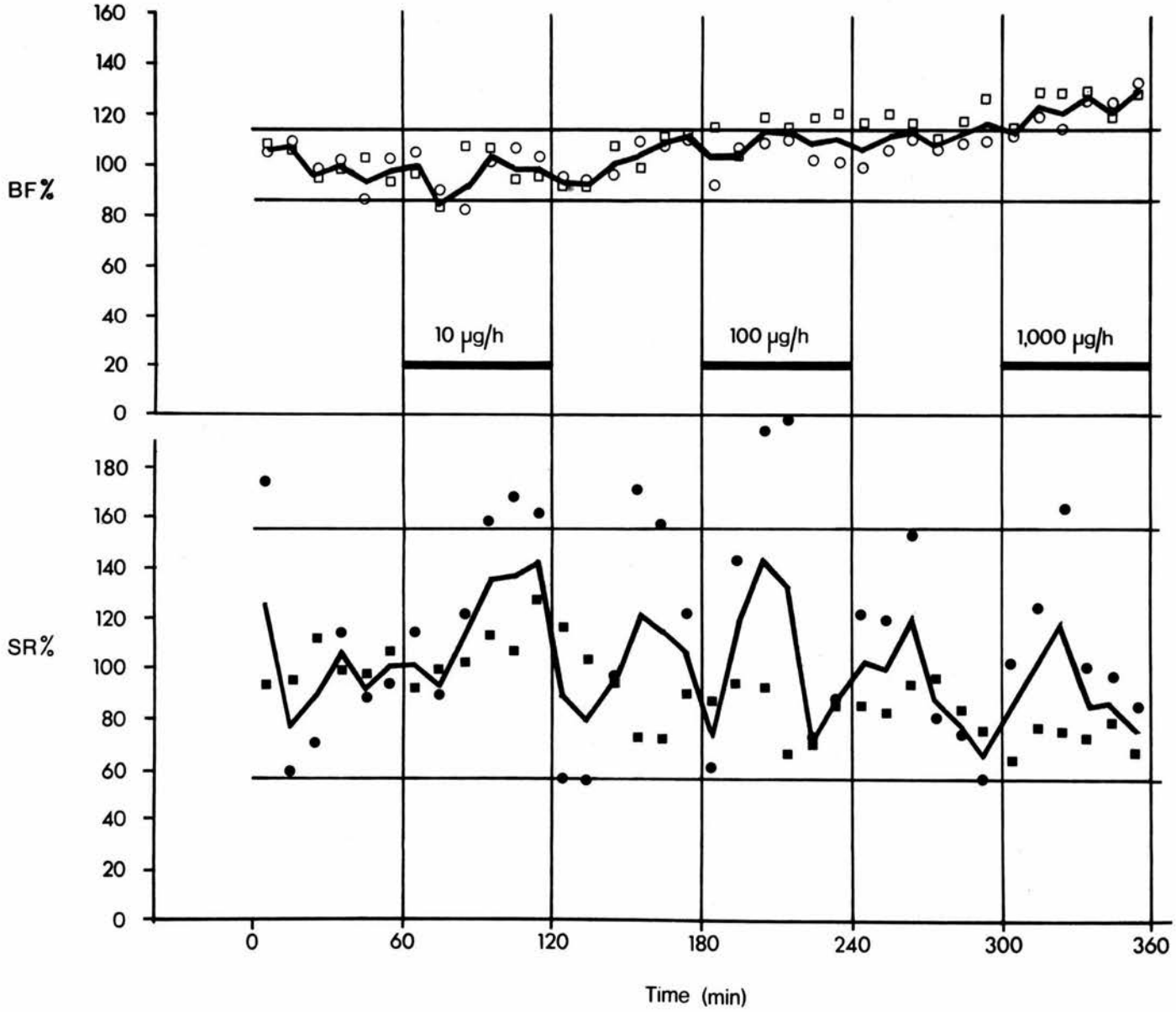
Mean relative changes in blood flow ( open symbols,) and in progesterone secretion rate ( solid symbols,) in two experiments ( experiment 18 circles, experiment 20 squares,) where maintained corpora lutea previously infused with LH were tested for their responses to three rates of LH infusion. Horizontal bars indicate the duration of LH infusions. The horizontal lines indicate the 95 % control limits for values observed during the first hour of each experiment.


BF%
SR%
160
140
120
100
80
60
40
20
0
180
160
140
120
100
80
60
40
20
0
10 µg/h
100 µg/h
1,000 µg/h
0
60
120
180
240
300
360
Time (min)

Legend to figure 29

Responses of maintained corpora lutea ( without prior luteinizing hormone infusion ) to luteinizing hormone infusion

Mean relative changes in blood flow ( open symbols,) and in progesterone secretion rate ( solid symbols,) in two experiments ( experiment 21 circles, experiment 23 squares,) where maintained corpora lutea not previously infused with LH were tested for their responses to three rates of LH infusion. Horizontal bars indicate the duration of LH infusions. The horizontal lines indicate the 95 % control limits for values observed during the first hour of each experiment.


BF%
SR%
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0
10 µg/h
100 µg/h
1,000 µg/h
0
60
120
180
240
300
360
Time (min)

Legend to figure 30

Responses of aged-maintained corpora lutea to luteinizing hormone infusion

Mean relative changes in blood flow ( open symbols,) and in progesterone secretion rate ( solid symbols,) in two experiments ( experiment 22 circles, experiment 24 squares,) where aged-maintained corpora lutea previously infused with LH were tested for their responses to three rates of LH infusion. Horizontal bars indicate the duration of LH infusions. The horizontal lines indicate the 95 % control limits for values observed during the first hour of each experiment.

Discussion

Intact ewes ovulate a day after the onset of oestrus, and full luteal activity is attained within eight or ten days ( see chapter 1 ). It is, therefore, likely that the ovaries perfused with LH 11 and 12 days after PG infusion correspond to those of normal ewes on days 9 and 10 of the cycle. This is the earliest stage at which the fully developed corpus luteum can be challenged with LH. The timing of the second LH infusion was chosen so that the LH treated corpora lutea would be similar to those borne by the sheep used in the experiments described in chapters 3 and 4. The time interval between LH infusions was maintained at about one month in the experiments on MX9 and D342 in order that the effect of prior LH infusion might be tested.

One ewe failed to return to oestrus after PG infusion, but this was not unexpected because the experimental ewes were taken from a flock which has a high proportion of 'silent'heats ( A.G. Wheeler, unpublished ).

The mean progesterone secretion rates observed during the first control period on the date of the first LH infusion differed from those observed during the equivalent period of the second LH infusions in all the ewes except D304 ( see table 11 ). However, it is assumed that the original corpora lutea were tested on the date of the second LH infusions, because work on similar ovarian autotransplanted ovaries confirmed that the corpora lutea of such animals are maintained for a long time ( see chapter 2 ).

When LH was infused at a rate of 1000 µg/h, the blood flow was significantly raised in 5 of the 8 experiments. However, that result may not be due to an action of LH on the ovary ( see chapter 2 ). Indeed, it is interesting to note that the ewe which failed to respond to LH by increasing its blood flow was the preparation with the lowest flow rate. Since the ovarian contribution in that preparation was likely to be a proportion of the total greater than that in the other three ewes, the increases in blood flow obtained may have been caused by an action of the infusate on non-ovarian capillary beds.

The conclusion that the older corpus luteum is more sensitive, and that the pretreated one is more responsive needs to be treated with caution. At each stage studied, only two animals were used, and in only one of the 4 pairs of experiments ( on maintained corpora lutea previously infused with LH ) were the changes in progesterone secretion rate observed in individual experiments fully consistent with each other.

The results in table 10 suggest that the states of follicular development were similar in each of the ovaries tested. It seems unlikely that the differences in luteal sensitivity and responsiveness shown in figures 27 to 30 could be caused by competition for LH between follicles and corpora lutea. If it is assumed that oestrogen secretion rates reflect intra-ovarian oestrogen concentrations, the different luteal responses observed in vivo cannot be ascribed to the inhibition of progesterone synthesis by oestrogen which has been demonstrated in vitro ( Talahay & Marcus, 1956; Cook, Niswender, Sutterlin, Norton & Nalbandov, 1968; Masaracchia & Gawienowsky, 1969; Akbar, Stormshak & Lee, 1972 ).

The ability of maintained, LH-pretreated corpora lutea to respond three times in quick succession to increasing doses of LH argues against the idea, advanced in chapter 4, that the ovary runs out of biosynthetic precursors.

The finding that old corpora lutea are more responsive than young ones is interesting. Possibly the ovulatory surge of LH at oestrus saturates the LH receptor sites ( Rajaniemi & Vanha-Perttula, 1972 ) rendering the luteal tissue refractory to further stimulation for a period of several days. This idea is supported by a single observation that follicular fluid may contain a high concentration of LH in the absence of a high plasma concentration ( Bjersing, Hay, Kann, Moor, Naftolin, Scaramuzzi, Short & Younglai, 1972 ). Perhaps the presence of high concentrations of LH bound to luteal tissue could explain the finding that young corpora lutea are much more resistant to hypophysectomy than are those late in the oestrous cycle, or those maintained in hysterectomized ewes ( Denamur, Martinet & Short, 1966 ).

The increased responsiveness observed after prior LH infusion can be explained by hormonally increased numbers of LH receptors, a phenomenon already noted in the rat ( Lee & Ryan, 1971 ). Alternatively, the first LH treatment may have induced steroid synthesizing enzymes ( Moor, Hay & Caldwell, 1972 ).

One aspect of luteal function is clearly illustrated in figures 27 to 30; in none of the 8 experiments was LH infusion able to induce a sustained and greatly increased progesterone secretion

rate. The pair of experiments conducted with ovaries corresponding most closely to those of intact sheep during the luteal phase of the oestrous cycle ( figure 27 ) hardly responded to LH. This result confirms the results of others who measured secretion rates at operation under anaesthesia ( Short, McDonald & Rowson, 1963; Domański, Skrzeczkowski, Stupnicka, Fitko & Dobrowolski, 1967; Cook, Kaltenbach, Niswender, Norton & Nalbandov, 1969; Hixon & Clegg, 1969 ) and who were unable to secure a secretion rate response to LH stimulation in the intact ewe. It now seems unlikely that their failure to observe a response was due to the use of anaesthesia.

In this series of experiments, the delivery of the stated doses of LH was confirmed by RIA. The inconsistent responses to dose rates below 1000 $\mu$g/h LH noted in chapter 3 were probably not explained by the absorption of some LH onto 'Millipore' filters.

Increased responsiveness towards gonadotrophin has been observed after pretreatment of bitches with trophic hormones ( Nishizawa & Eik-Nes, 1964 ). It is, therefore, probable that the increased rate of progesterone secretion seen previously during LH infusion ( McCracken, Uno, Goding, Ichikawa & Baird, 1969; chapter 3 ) is an abnormal response characteristic of ovaries pretreated with large doses of gonadotrophin. That conclusion is supported by a recent demonstration that the enzyme content and distribution in ovaries of ewes treated with PMSG are abnormal ( Moor, Hay & Caldwell, 1972 ).

CHAPTER SEVEN

# THE EFFECTS OF CYCLIC NUCLEOTIDES AND AMINOPHYLLINE

## Introduction

Intensive biochemical investigations into the actions of adrenaline - primarily on heart muscle and adipose tissue - and into the action of glucagon on the liver led to the formulation of a general theory of hormone action ( Sutherland, Øye, Butcher, 1965 ). They proposed that hormones interact at specific membrane sites on target tissue cells. There, a 'second messenger' common to the different tissues is formed. That messenger was identified with cyclic-3',5'-adenosine monophosphate, and it was suggested that this compound could interact with intracellular enzymes to produce the biochemical changes characteristic of hormone stimulated cells. Since that date, cyclic adenosine monophosphate ( cAMP ) has been implicated in very many regulatory processes ( see Major & Kilpatrick, 1972 ).

Studies into the mechanism of hormone action have been extended to the effects of LH on the ovary. Most of these studies have been in vitro, and many used sliced luteal tissue especially from the cow. LH was found to stimulate luteal adenyl cyclase

causing intracellular concentrations of cAMP to rise; later steroid secretion rose. Exogenous cAMP was found to mimic the effect of LH on steroid synthesis ( Savard, Marsh & Rice, 1965; Marsh, Butcher, Savard & Sutherland, 1966 ). More recently, the action of LH and FSH on prepubertal rat ovarian tissue *in vitro* has been shown to be mimiced by cAMP ( Ahrén, Hamberger & Rubenstein, 1969 ). Using rabbit tissue, a similar result was obtained ( Dorrington & Kilpatrick, 1969 ). The last group showed that phosphodiesterase inhibitors - methylxanthines such as theophylline - potentiated the action of concentrations of LH and cAMP that by themselves failed to effect a maximal response. Furthermore, dibutyryl cAMP, like LH, can induce luteinization of cultured granulosa cells ( Channing, 1970 ). All these studies suggest that cAMP is the 'second messenger' in the action of LH on ovarian tissue.

Few workers have attempted to confirm that the effects seen *in vitro* also occur *in vivo*. Two approaches are possible: in the first, cAMP is infused or injected systemically; in the second, nucleotide is infused close intra-arterially into the organ being studied. An example of the first is provided by a group investigating cortisol secretion ( Angeli, Boccuzzi, Frajria, Bisbocci & Ceresa, 1973 ). The disadvantages of this approach are apparent from the number of side effects suffered by the experimental subjects. Theophylline injections into immature rats deplete ovarian ascorbic acid ( Sowerby-Cooper & Lunn, 1969 ), but the investigators were not able to conclude that the result was a direct effect on the ovary because cAMP is probably involved in the

secretion of pituitary hormones ( see Major & Kilpatrick, 1972 ). An action of cyclic nucleotide *in vivo* has been demonstrated during close intra-arterial infusion in the testis ( Connell & Eik-Nes, 1969 ), the thyroid ( Ahn, Athans & Rosenberg, 1969 ), and in thyroid C cells ( Care, Bates & Gitelman, 1970 ). To the author's knowledge, no attempt to show an effect of cyclic nucleotide on the ovary during close intra-arterial infusion has yet been reported.

The experiments in this chapter were performed in order that the effects of cAMP noted *in vitro* might be tested *in vivo*.

Materials and methods

Adenosine-3',5'-cyclic monophosphoric acid was purchased from British Drug Houses ( batch 1155390 ). On the day before use a solution of the required concentration was prepared in sterile saline. The solution was stored overnight at 4°C.

$N^6$-2'O-dibutyryl-adenosine-3',5'-cyclic monophosphate ( dbcAMP ) was obtained as the monosodium salt from Sigma ( batch 916-7020 ). The salt was dissolved in sterile saline on the day of use because the compound is reported to undergo decomposition in solution ( Swislocki, 1970 ).

Aminophylline ( Am ) was bought from Sigma ( batch 31C-2200 ). The solutions were prepared in sterile saline on the day of use. Aminophylline is the ethylene diamine salt of theophylline. It is more soluble in water than theophylline, and comprises 85 % by weight theophylline.

Table 12.

Summary of experiments involving cyclic nucleotide infusions

| Expt. No. | Ewe No. | Date | Compound infused | Rate mg/min | Duration min | Prior infusions on the same day | Previous experiments |
|---|---|---|---|---|---|---|---|
| E | D342 | 25-3-70 | cAMP | 0.01<br>0.1 | 30<br>30 | 0.05 mg LH | Experiment D two weeks earlier 0.05 mg LH |
| 2 | CB69 | 23-4-70 | dbcAMP | 0.01<br>0.1 | 55<br>55 | 1 mg LH | Experiment C one month earlier 0.01 mg LH |
| 3 | D342 | 30-4-70 | dbcAMP | 0.1 | 2x30 | 0.8 mg LH | Experiment E one month earlier 0.05 mg LH |
| 4 | CB94 | 28-5-70 | dbcAMP | 0.05 | 60 | 1 mg LH | PMSG & HCG treatment December 1969 |
| D | D342 | 11-3-70 | cAMP | 0.15<br>0.3 | 30<br>30 | see fig. 31a. | none |
| 5 | D342 | 2-7-70 | dbcAMP | 0.3 | 60 | see fig. 31b. | Experiment 3 two months earlier 1 mg LH |
| 6 | D342 | 10-9-70 | dbcAMP | 6.4 | 8 | see fig. 31c. | Experiment 5 two months earlier 1 mg LH |

Table 13.

Summary of experiments involving aminophylline infusion

| Expt. No. | Ewe No. | Date | Details | Mean SR*[a] 1st. period | Mean BF*[b] 1st. period | Previous experiments |
|---|---|---|---|---|---|---|
| 25 | MX9 | 21-1-71 | fig. 32 | 3.2 ± 0.83 | 20.2 ± 3.07 | None; ewe showed heat 8-9-71 after PG induced luteolysis. |
| 26 | D304 | 9-10-72 | fig. 32 | 0.45 ± 0.054 | 18.0 ± 1.52 | None; ewe showed heat 30-8-72 after PG induced luteolysis. |
| 27 | D305 | 21-12-72 | fig. 33 | 3.2 ± 0.55 | 13.4 ± 0.76 | None; ewe had low peripheral progesterone on 4-11-72 after PG induced luteolysis. |
| 28 | D314 | 14-2-73 | fig. 33 | 3.7 ± 0.53 | 16.0 ± 0.76 | 1 mg LH 19-12-72. Ewe showed heat 7-12-72 after PG induced luteolysis. |

*Mean ± standard deviation. [a] µg / min [b] ml / min

Ewes with autotransplanted ovaries were prepared for intra-arterial infusion and collection of ovarian vein blood by Dr. D.T. Baird as described in chapter 2. Blood collections were taken by Dr. D.T. Baird in the experiments listed in table 12 and by the author in those experiments listed in table 13.

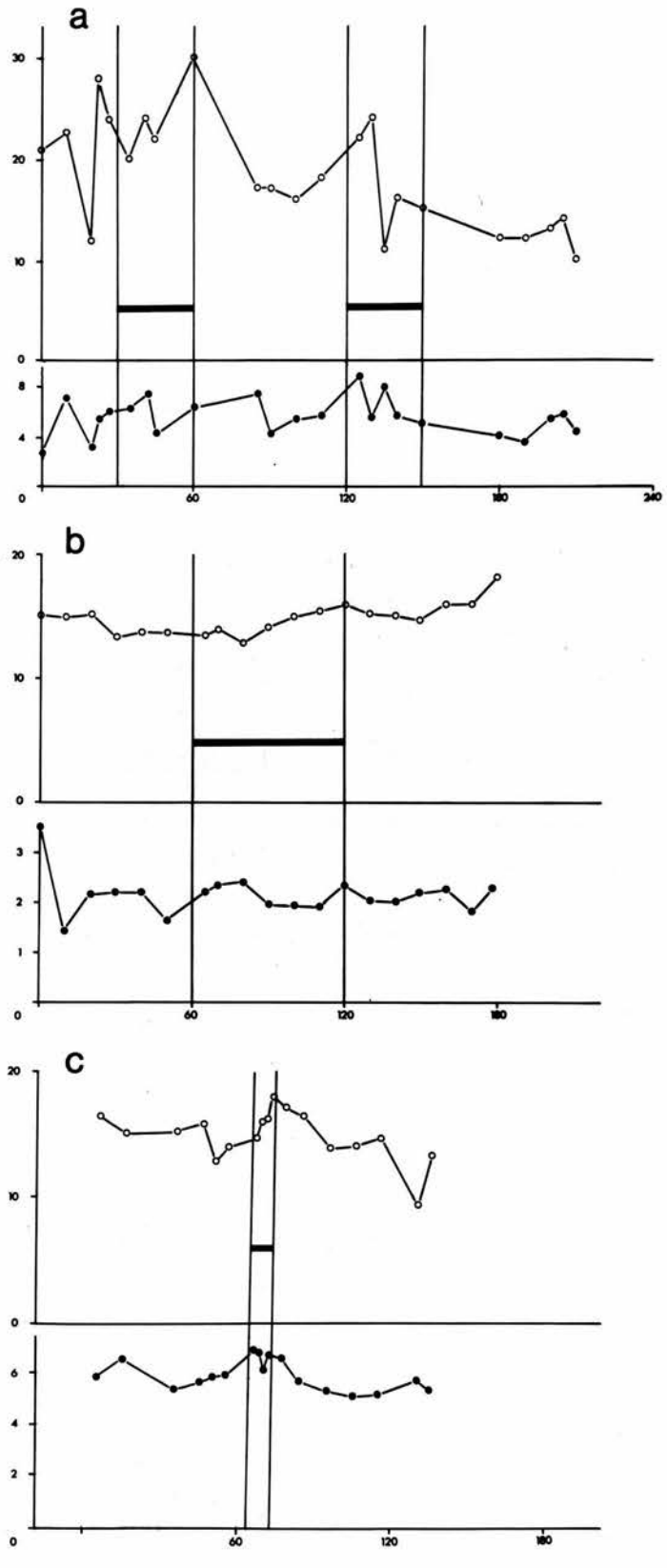
Progesterone concentrations in samples of ovarian vein plasma were estimated according to table 1. Calculations and statistical tests were applied as described in chapter 2.

Tables 12 and 13 summarize the experiments discussed in this chapter. After experiment E, dbcAMP was used instead of cAMP because the derivative penetrates cell walls more readily, and because it is more resistant to the action of phosphodiesterases ( Posternak, Sutherland & Henion, 1962 ). Furthermore, Channing ( 1970 ) reports that dbcAMP is one hundred times more active than cAMP in causing luteinization of granulosa cell cultures.

After the experiments in table 12 had been completed, other experiments had shown that the ovary became refractory to further stimulation after prior LH stimulation ( chapter 4 ). A series of four experiments where no LH was infused on the day of dbcAMP infusion were conducted.

Results

LH was infused before cyclic nucleotide in experiments E, 2, 3 and 4 ( see table 12 ). No increases in progesterone secretion rate occurred during cAMP or dbcAMP infusion. Blood flow


a
30
20
10
0
8
4
0
60
120
180
240
b
20
10
0
3
2
1
0
60
120
180
c
20
10
0
6
4
2
0
60
120
180

Legend to figure 31

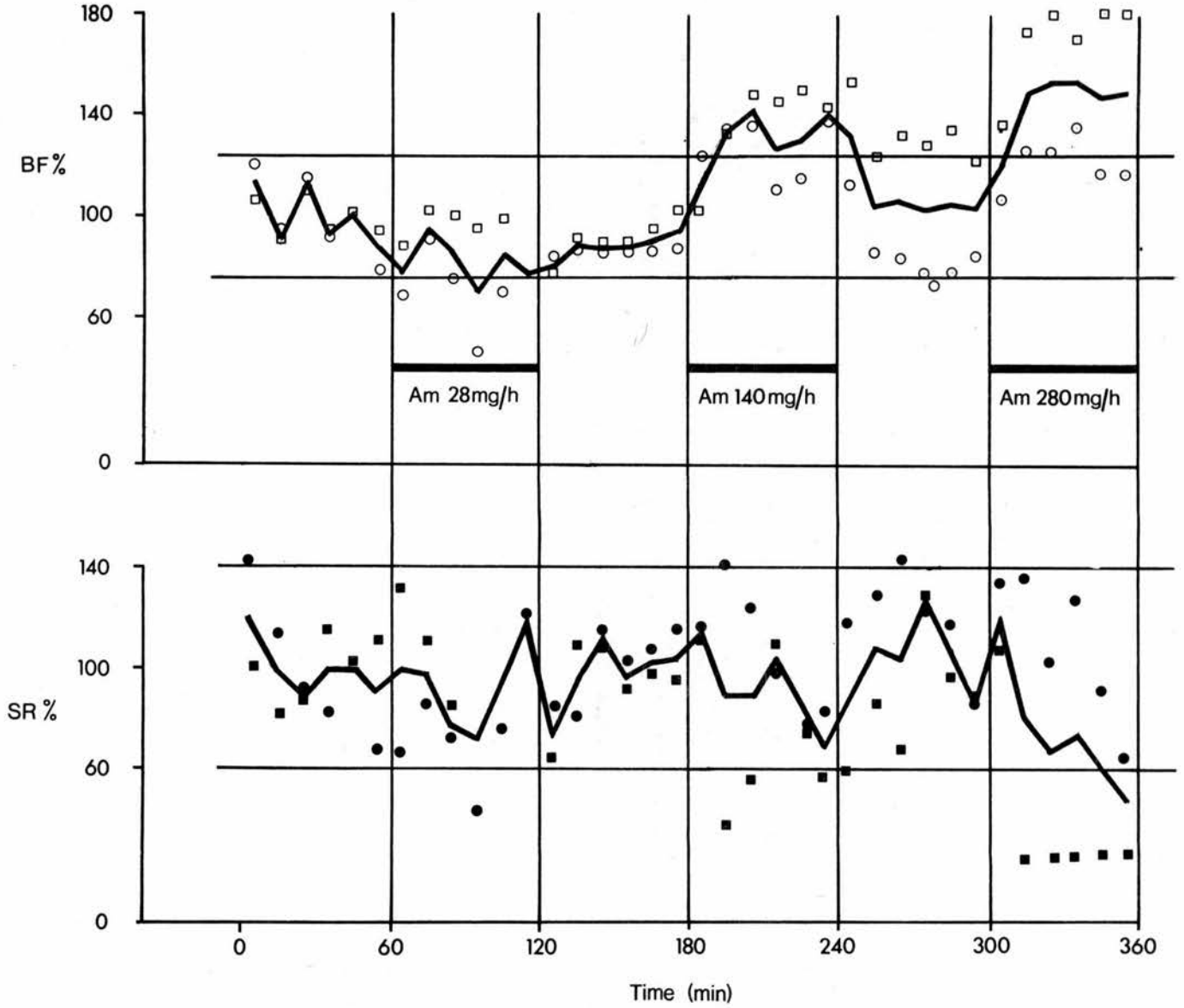
Responses to cAMP and dbcAMP infusion

Changes in blood flow ( open circles, ml / min ) and in progesterone secretion rate ( solid circles, µg / min ) in three experiments where cyclic nucleotides were infused. The horizontal axes indicate the time in minutes. The horizontal bars show the duration of nucleotide infusions.

a), experiment D: cAMP infused at the rates of 150 and later 300 µg / min.

b), experiment 5: dbcAMP infused at the rate of 300 µg / min.

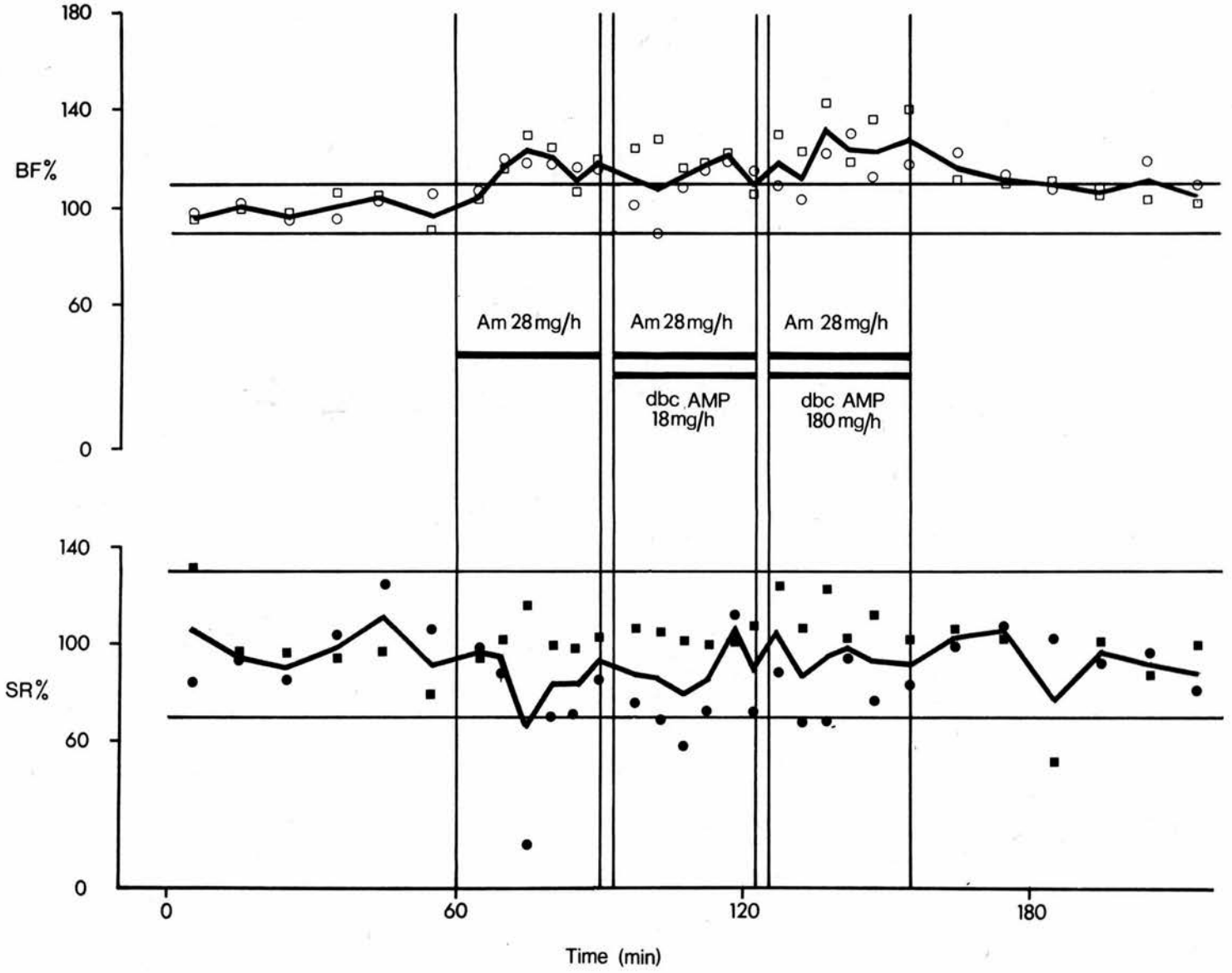
c), experiment 6: dbcAMP infused at the rate of 6.4 mg / min.


180
140
100
60
0
BF%
Am 28mg/h
Am 140mg/h
Am 280mg/h
140
100
60
0
SR %
0
60
120
180
240
300
360
Time (min)

Legend to figure 32

Responses to aminophylline infusion

Mean relative changes in blood flow ( open symbols,) and in progesterone secretion rate ( solid symbols,) in two experiments ( experiment 25 circles, experiment 26 squares ) where three doses of aminophylline ( Am ) were infused. Horizontal bars indicate the duration of periods of aminophylline infusion. Horizontal lines show the 95 % control limits for values observed during the first hour of the experiments.


180
140
BF%
100
60
0
Am 28mg/h
Am 28mg/h
Am 28mg/h
dbc AMP
18mg/h
dbc AMP
180mg/h
140
100
SR%
60
0
0
60
120
180
Time (min)

Legend to figure 33

Responses to simultaneous aminophylline and dbcAMP infusion

Mean relative changes in blood flow ( open symbols,) and in progesterone secretion rate ( solid symbols,) in two experiments ( experiment 28 circles, experiment 27 squares ) where aminophylline ( Am ) and dbcAMP were infused. Horizontal bars indicate the duration of periods of nucleotide or aminophylline infusion. The horizontal lines show the 95 % control limits for values observed during the first hour of the experiments.

rose progressively throughout the nucleotide infusions in experiments E, 2 and 5. The results of experiments D, 5 and 6 are shown in figure 31.

Figure 32 shows the results of a pair of experiments in which aminophylline was infused. The highest dose rate ( 280 mg/h ) caused a cessation of progesterone secretion in experiment 26. in experiment 25 secretion rates were variable. The changes in blood flow were similar in both experiments. Rises occurred during the period of infusion of the two higher dose rates.

Figure 33 shows the results of experiments 27 and 28 in which dbcAMP was infused with aminophylline. In neither experiment was an increase in secretion rate seen, but blood flow increased after the first control period.

## Discussion

### Nucleotide infusions

The failure of cAMP and dbcAMP infusions to increase progesterone secretion in four experiments ( E, 2, 3 and 4; see table 12 ) may have resulted from refractoriness induced by prior LH infusion ( chapter 4 ). However, this objection does not apply to two further experiments ( D and 5; see figure 31 ). The dissociation of the steroid secretion response from the blood flow response is illustrated by experiments E, 2 and 5. In experiment 5, the concentration of nucleotide in the blood perfusing the ovary was about 0.05 mM, a concentration much lower than those used *in vitro* ( Savard, Marsh & Rice, 1965 ) or *in vivo* ( Connell & Eik-Nes, 1969 ).

In a subsequent experiment, a higher dose rate of dbcAMP was employed. In experiment 6 the blood nucleotide concentration was about 1 mM. A slight increase in secretion rate was observed ( see figure 31c ). However, it was of short duration, and at no previous control period have blood collections been taken so frequently. It is, therefore, quite impossible to exclude slight changes in endogenous secretion rate of this limited duration. It cannot be taken as a significant response.

Antiphosphodiesterase infusions

When these experiments had been completed, it was thought possible that cyclic nucleotide is destroyed in the blood by phosphodiesterases. In four experiments summarized in table 13, a phosphodiesterase inhibitor was infused alone, or simultaneously with dbcAMP. The highest rate of aminophylline infusion is likely to lead to a concentration of 2 mM in the blood perfusing the ovary. The results of these four experiments are shown in figures 32 and 33.

At high dose rates, aminophylline infusion leads to an immediate increase in blood flow. The pattern is unlike that previously observed during LH ( chapter 3 ) or dbcAMP infusion ( see figure 31b ). Possibly the mechanism of action of aminophylline is unlike that of LH or dbc AMP.

The abrupt cessation of progesterone secretion brought about by aminophylline infusion in experiment 26 ( see figure 32 ) was surprising. Perhaps the ethylene diamine in aminophylline is able to act as a chelating agent to sequester calcium ions in the cells of luteal tissue. It has long been known that adenyl

cyclase requires calcium for activity ( Sutherland, Rall & Menon, 1962 ). More recently, ACTH activation of adrenal adenyl cyclase has been shown to require calcium ions ( Lefkowitz, Roth & Pastan, 1970 ), and it has been proposed that prostaglandins exert their effects by way of displacing calcium ions from cell membranes ( Ramwell & Shaw, 1970 ). The luteolytic activity of aminophylline in one experiment is like that of prostaglandin $F_{2\alpha}$ *in vivo* ( Chapter 5 ). Opposed to these speculations are the findings of Channing ( 1970 ); she showed that aminophylline increased progestin secretion from cultured granulosa cells.

Simultaneous nucleotide and antiphosphodiesterase infusions

It was concluded from experiments 25 and 26 that infusion of 28 mg/h aminophylline had no effect on blood flow or secretion rate. Accordingly, that rate of infusion was adopted in experiments 27 and 28. Unfortunately that dose rate did increase blood flow in these two experiments ( see figure 33 ). The infusion rate of dbcAMP should have been sufficient to maintain a concentration of about 0.5 mM in the blood perfusing the ovary. The failure to obtain an increased rate of progesterone secretion may still be attributed to an inadequate level of the nucleotide since this is below those used *in vitro* ( Savard, Marsh & Rice, 1965 ).

CHAPTER EIGHT

# GENERAL CONCLUSIONS

In this chapter, the findings described in the preceding sections are reviewed in the light of work by others. In particular, the rôle of pituitary hormones in luteal maintenance in the ewe is discussed. The strengths and weaknesses of the ovary transplant preparation are reviewed and some future developments are indicated.

## Ovine luteotrophic hormones

Although LH produces some increase in progesterone secretion from PMSG and HCG treated ovaries ( McCracken, Uno, Goding, Ichikawa & Baird, 1969 ), and a short-lived response from ovaries bearing maintained corpora lutea ( chapter 3 ), it is unable to stimulate progesterone secretion from corpora lutea ten days after oestrus ( chapter 6 ). The previous negative reports on the effect of LH at operation in the intact animal were therefore confirmed ( chapter 6 ).

An inability to stimulate luteal steroid secretion is not a new situation for a luteotrophic hormone. Prolactin fails to stimulate progesterone secretion from the rat ovary at

operation ( Yoshinaga, Grieves & Short, 1967 ). In chapter 6 it was suggested that normal corpora lutea of the cycle were already maximally stimulated by the low endogenous levels of pituitary hormones in the blood. The necessity for pituitary support is indicated by the results of hypophysectomy. Corpora lutea regress early if this operation is carried out just after oestrus ( Denamur, Martinet & Short, 1966 ), and the regression is prompt after hypophysectomy of hysterectomized ewes bearing maintained corpora lutea. The need for LH is demonstrated by experiments in which the administration of antiserum to LH terminated luteal function in ewes ( Dermody & Foote, 1968; Fuller & Hansel, 1970; McCracken, Baird & Goding, 1971 ). Recent work shows that LH is a component of the ovine luteotrophic complex because it is required for normal luteal function in hysterectomized hypophysectomized ewes ( Denamur, Martinet & Short, 1973 ).

The rôle of prolactin as a luteotrophic hormone in animals other than rodents was generally discounted. The addition of prolactin to incubations of luteal tissue in vitro had no effect on steroid synthesis ( Kaltenbach, Cook, Niswender & Nalbandov, 1967 ). In vivo no significant rises in progesterone secretion occurred during prolactin administration to intact ewes at operation ( Short, McDonald & Rowson, 1963; Domański, Skørzeczkowski, Stupnicka, Fitko & Dobrowolski, 1967; Cook, Kaltenbach, Niswender, Norton & Nalbandov, 1969; Hixon & Clegg, 1969 ). Prolactin infusion has no effect on the autotransplanted ovary ( see chapter 4 ). Prolactin fails to delay luteal regression ( Karsch, Cook, Ellicott, Foster, Jackson & Nalbandov, 1971 )

unless it is mixed with LH in which case the treatment is sometimes effective ( Moore & Nalbandov, 1955; Karsch, Cook, Ellicott, Foster, Jackson & Nalbandov, 1971 ). The deficiency of this type of test is that the uterine luteolytic effect overrides the pituitary hormones ( see chapters 1 and 3 ).

In spite of these discouraging results with prolactin, it was known that pituitary-stalk-sectioned, hysterectomized ewes showed slower luteal regression than completely hypophysectomized ones ( Denamur, Martinet & Short, 1966 ). Furthermore, prolactin increases progesterone secretion from the ovary of acutely hypophysectomized ewes ( Hixon & Clegg, 1969 ). In a large series of experiments, it was demonstrated that both LH and prolactin were required for normal luteal weight and function in chronically hypophysectomized ewes ( Denamur, Martinet & Short, 1973 ).

The apparent discrepancy between the luteotrophic properties of prolactin in hypophysectomized ewes, and the inability of this hormone to stimulate luteal progesterone secretion from pituitary-intact ewes can be resolved by recent discoveries about the rôle of prolactin in laboratory animals.

In neither rats ( Marsh, Telegdy & Savard, 1966; Yoshinaga, Grieves & Short, 1967 ), nor in rabbits ( Hilliard, Endröczi & Sawyer, 1961 ), does prolactin increase progesterone secretion *in vivo*. However, prolactin does reverse the cholesterol ester depleting action of LH ( Armstrong, 1968; Hilliard, Spies & Sawyer, 1969 ) by increasing the activity of enzymes

concerned with cholesterol ester metabolism ( Armstrong, 1968; Behrman, Orczyk, McDonald & Greep, 1970 ). An additional effect of prolactin is to abolish ovarian refractoriness induced by prior LH treatment ( Armstrong, 1968; Hilliard, Spies & Sawyer, 1969 ). It may be concluded that LH stimulates progesterone secretion when cholesterol stores are high, and that prolactin maintains the level of those stores. Both LH and prolactin are required for maximal progesterone secretion. A similar situation has been discovered in the testis in which prolactin restores cholesterol ester levels after LH induced depletion ( Bartke, 1971 ), and in which prolactin and LH are required for maximum testosterone secretion in the hypophysectomized male rat ( Hafiez, Lloyd & Bartke, 1972 ). Prolactin may play a similar supporting rôle in large animals because prolactin increases progesterone secretion from LH-stimulated perfused bovine ovaries in vitro ( Bartosik & Romanoff, 1969 ).

In view of these findings, it is possible to propose a new test for luteotrophic hormones. They should restore ovarian cholesterol levels after LH induced depletion, and they should restore the ability of the ovary to respond to a further stimulus of LH. It is disappointing that further work was not done on the effects of repeated LH infusions ( chapter 4 ), because the autotransplant preparation is an ideal system for testing the luteotrophic properties of prolactin in the ewe by this new method.

Can over-stimulation produce luteolysis ?

In some experiments, precipitous falls in progesterone secretion rate were recorded ( chapter 4, experiment 10; chapter 7, experiment 26 ). Less dramatic declines occurred when prostaglandin $F_{2\alpha}$ was infused ( chapter 5 ). Although the sharp declines noted in chapters 5 and 7 were not consistent findings, and it is possible that they were caused by the accidental infusion of a small air bubble which later lodged in an arteriole supplying part of the corpus luteum, it is perhaps worth speculating on the consequences of over-stimulation of the gland.

In chapter 6, it was suggested that the corpus luteum was probably already maximally stimulated by endogenous LH. In such a situation, it is easy to envisage further stimulation leading to metabolic derangement and luteolysis in the same way that plant growth hormones act as weed killers when they are in high concentrations.

Intracellular concentrations of steroid intermediates may become inhibitory. For example, pregnenolone inhibits side-chain cleavage of cholesterol in adrenal tissue ( Koritz & Hall, 1964 ). Possible modes of product inhibition have been considered by Armstrong ( 1968 ).

High concentrations of 'second messengers' may also be inhibitory. A regulatory role has already been proposed for cAMP because it retards the adrenal conversion of pregnenolone to

progesterone *in vitro* ( Berger, 1971 ). LH is claimed by one group to act *via* intracellular prostaglandin $F_{2\alpha}$ ( Kuehl, Humes, Tarnoff, Cirillo & Ham, 1970 ). That claim is supported by a further report that shows prostaglandin to stimulate adenyl cyclase in ovarian tissue ( Marsh, 1970 ). Moreover, prostaglandin $F_{2\alpha}$ release has been detected from ovarian tissue after stimulation with LH *in vitro* ( Chasalow & Pharriss, 1972 ). Thus over-stimulation could result in a high concentration of prostaglandin $F_{2\alpha}$ a substance which has a profound effect on cholesterol ester metabolism ( Behrman & Greep, 1971 ).

This over-stimulation hypothesis explains why prostaglandin $F_{2\alpha}$ generally stimulates steroidogenesis *in vitro* ( in a gonadotrophin free environment ), whereas it is luteolytic *in vivo* ( see chapter 5 ). Rather more recently, Behrman in discussion after a paper on prostaglandins and hormone action ( Pharriss, Tillson & Erickson, 1971 ) has been able to show that prostaglandin $F_{2\alpha}$ increases steroidogenesis *in vivo* in a hypophysectomized animal. These findings are consistent with others showing that very low amounts of prostaglandin $F_{2\alpha}$ increase steroid secretion from the transplanted ovary ( McCracken, Glew & Levy, 1970 ). The hypothesis also explains why LH is unable to protect the ovary from the effects of prostaglandin $F_{2\alpha}$ ( Cerini, Chamley, Findlay, Goding, 1973 ).

The future for the ovary transplantation preparation

One of the major difficulties of this preparation is the variation which is encountered both between and within infusions ( see chapter 3 ). It was not possible to relate that variation

to the time in the breeding season, the animal used, or the experimenters performing the blood collections or the cannulations. Unless the sources of this variation are discovered, it will be impossible to conduct elegant dynamic studies, such as those reported on the adrenal ( Urquhart & Li, 1968 ).

An important finding was that there were severe limitations on the use of each animal 'as its own control' ( McCracken & Baird, 1969 ). Chapter 4 shows that the ovary fails to recover, at least in the period of two hours, from gonadotrophic stimulation. Thus an identical stimulus fails to produce the same effect. Chapter 6 indicates that the ovary may not recover its initial state even after one month.

Ovarian function in the autotransplant preparation has been reviewed ( Goding, Baird, Cumming & McCracken, 1972 ). It is possible that transplantation has altered the ovary in four important ways. Severing the normal connexions between ovary and uterus leads to prolonged luteal maintenance, and this has been considered in chapter 2. It had been suggested that the exposed position of the transplanted organ could lead to abnormally low intra-ovarian temperatures, and so alter ovarian steroidogenesis. The effect of temperature on the ovary has been investigated ( McCracken, Baird & Goding, 1971 ), and it was concluded that the very small difference in temperature that exists between the abdomen and skin loop is unlikely to influence ovarian steroid secretion significantly.

In transplanting any organ, the nerves are cut. If, as has been suggested, the control of ovarian function is by way of the nervous system ( Moore & Nalbandov, 1953; Restall, Hearnshaw, Gleeson & Thorburn, 1973 ), then this becomes of great importance. However, it is possible for transplanted organs to regain a nervous supply ( Goding, Baird, Cumming & McCracken, 1972 ).

The most serious criticism of the ovary transplant preparation centres on the nature of the measured blood flow. This matter was discussed in chapter 2 where I concluded that changes in blood flow observed from this preparation were probably unrelated to ovarian function. This conclusion is opposite to that reached in a recent review ( Goding, Baird, Cumming & McCracken, 1972 ).

A number of modifications have been proposed for the transplantation procedure in order that some of the criticisms might be countered. Results of work on the utero-ovarian transplant, in which normal connexions between the uterus and ovary are retained, have already been published ( McCracken, Glew & Levy, 1970; McCracken, Baird & Goding, 1971 ). They show that normal cyclical function is retained ( see also chapter 2 ).

The problem of variability has been touched on already. One factor making ovarian vein blood collections difficult is the close juxtaposition of the carotid artery and the jugular vein. It is not always easy to occlude the vein completely while still leaving the artery patent. Dr. D.T. Baird has recently prepared ewes with skin loops divided at their caudal end so that

one limb receives the jugular vein, and the other, the carotid artery. Taking complete ovarian vein blood collections from these ewes is much simpler than from ewes with undivided loops.

A likely source of variation is from the endogenous pituitary hormones. These could be eliminated by the difficult operation of hypophysectomy. However, were that operation to be successful, the ovary would atrophy unless maintenance therapy were started. That procedure would require large amounts of purified gonadotrophins, and it would not be possible to supply them in the same way that they are released from the pituitary. A more practical approach would be to monitor the endogenous gonadotrophin levels. Unfortunately, the low concentrations of LH at dioestrus are at, or below the limit of sensitivity of existing immunoassay methods.

The merits of the preparation have to be considered alongside those of other techniques. The important advantage of the preparation is to provide long term access to the ovary. The studies reported in chapters 5 and 6 could not have been done by any other existing method. Those studies in other chapters would be most difficult to do by <u>in vitro</u> perfusion.

The preparation has already yielded much useful information about the luteolytic properties of prostaglandins. Other pharmacological agents could be screened for their direct effects on the ovary. Of particular interest would be enzyme inhibitors such as aminoglutethimide and AY9944, and substances like clomiphene.

The events surrounding ovulation and oestrus are of economic importance in sheep breeding. Utero-ovarian transplants could be used to study these problems. It has been reported that multiple ovulations are more frequent when sheep are on good pasture ( McKenzie & Terrill, 1937 ). The incidence of oestrus is also said to be increased by 'flushing' ( Marshall, 1903; McKenzie & Terrill, 1937 ). The preparation could allow a closer look at the effect of such environmental influences on ovarian function. The effects of different thyroid and adrenal conditions on reproduction might also be studied with the autotransplant preparation.

Perhaps one of the most interesting problems for the reproductive endocrinologist is the transformation which occurs at puberty. If it were possible to transplant the immature ovary of a ewe lamb, ovarian function at puberty could be closely followed.

Use of one technique alone will not unravel the complexities of the ovary. Several different approaches must be explored. Enzyme levels have to be studied by in-vitro methods, and intra-ovarian concentrations of substances may have to be determined by methods that destroy the tissue. Neverteless I conclude that the preparation will be an important tool for endocrinologists for years to come.

CHAPTER NINE

# SUMMARY

A better understanding of the processes involved in reproduction is likely to aid the development of methods for regulating the fertility of humans and animals. Ultra-sensitive and rapid assays for steroids, and a long term surgical preparation of the sheep ovary have been recently described. The advent of these two techniques makes it possible to investigate the action of trophic hormones on ovarian steroid secretion in vivo in greater detail than has been attempted before.

A method for assaying progesterone in ovine peripheral, or ovarian vein plasma was developed by modifying previously published procedures. The specificity, accuracy and precision of the method were investigated. They were judged to be satisfactory in relation to the purpose for which the assay was used, and in relation to the performance of other, published progesterone assays.

The function of the transplanted sheep ovary was reviewed, and it was confirmed that extended luteal function in the transplanted organ was caused by separation of the ovary from the uterus. In one experiment, it was concluded that the blood

flow from the preparation was not a reliable indication of ovarian blood flow. The intra-arterial infusion of non-specific proteins had little effect on progesterone secretion rate from the transplanted ovary.

The effects of close intra-arterial infusion of LH were studied. In spite of much individual variation, it was concluded that the use of saturating doses of LH produced only a temporary rise in progesterone secretion. The pattern of this response was characterized.

Human chorionic gonadotrophin had an effect indistinguishable from that of LH. After stimulation by either of these hormones, the ovary becomes refractory towards further stimulation. The hypothesis that LH had depleted luteal cholesterol ester - a precursor for progesterone synthesis - was considered a possible explanation for this phenomenon. Two experiments with prolactin - which is reported to restore ovarian cholesterol stores after LH induced depletion - were inconclusive.

The luteolytic action of prostaglandin $F_{2\alpha}$ was confirmed *in vivo*. It was concluded that prostaglandin infusion was a reliable way of regressing maintained *corpora lutea* and thereby inducing the formation of a new one after ovulation at oestrus.

The responsiveness of *corpora lutea* was found to vary with age and previous hormone treatment. Day 10 *corpora lutea* were insensitive to LH, whereas older maintained *corpora lutea*

were sensitive, and they responded by increasing their secretion of progesterone. Variations in endogenous oestrogen secretion were not responsible for this difference. It was concluded that the greater sensitivity of luteal tissue induced by hormone treatment was not typical of the normal corpus luteum of the ovine oestrous cycle.

Cyclic adenosine-3',5'-monophosphoric acid and a more active derivative were found not to increase progesterone secretion when they were infused into the transplanted ovary. Aminophylline also failed to produce a consistent effect.

The rôle of prolactin and LH as luteotrophic hormones in the ewe was reviewed in the light of their failure to increase long term progesterone secretion from the normal corpus luteum in vivo. Some speculation on the biochemical mechanism of luteolysis was advanced.

It was concluded that the ovarian transplant preparation would continue to have an important place in studies on the long term alterations in luteal function induced by drugs or environmental changes. The preparation is less suitable for studying intracellular ovarian events, or the events around ovulation; it is unsuitable for studying ovarian blood flow. A number of modifications of the preparation were proposed, and the more complex utero-ovarian transplant was considered to have great promise. It is clear that these preparations will be studied for years into the future, and it is hoped that the work in this thesis will form part of the basis for future advances in this field.

# APPENDIX A

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